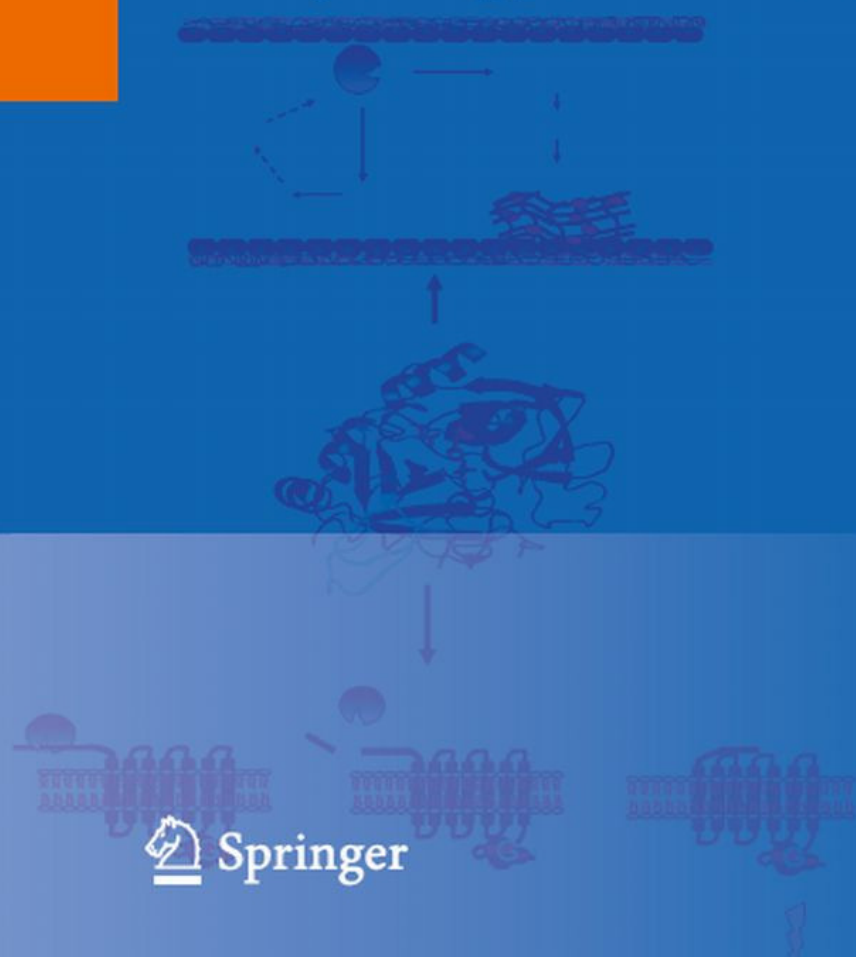


Michael E. Maragoudakis
Nikos E. Tsopanoglou
Editors

Thrombin: Physiology and Disease



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Michael E. Maragoudakis • Nikos E. Tsopanoglou
Editors

Thrombin

Physiology and Disease



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Preface

The elucidation of the coagulation cascade has been one of the major accomplishments of biomedical sciences in recent years. The central role of thrombin, the plethora of factors and the multiple control mechanisms involved in haemostasis have been well characterized. However, new insights in the structure, functions and regulatory roles of thrombin in vascular physiology and development, in neuronal system, in tumor biology, tissue repair and angiogenesis have been gained by new powerful techniques and pioneering work of leading scientists.

In the chapters of this book it was attempted to provide a comprehensive present day view of thrombin, as an enzyme in relation to blood coagulation and in relation to its receptors. The effects of thrombin on various cell types and in patho-physiological conditions are discussed. This book is written to integrate the current understanding of thrombin basic mechanisms in vascular, inflammatory, neuronal and tumor cells and molecular biology with the experimental and clinical implications of these advances. Our goal was not to provide an exhaustively referenced compendium of the many topics that touch upon thrombin functions. Instead, we sought to create a practical and concise summary that would be equally useful for those first entering the field and for those with expertise in one facet of thrombin function wishing to learn more about others. Special emphasis was given to the new roles of thrombin and its receptors in vascular and tumor biology as well as angiogenesis, which present a challenge to translate this knowledge to therapeutic targets

We believe that this book will solidify the concept that thrombin and its receptors are a dynamic, vibrant field with clear implications for understanding the pathogenesis of several diseases (cardiovascular, neuronal, cancer) and for the treatment of patients. We hope that it will serve as a useful resource, not only for those involved in education and in the pursuit of new research programs, but also for those caring for patients with above diseases.

Patras, Greece
May 2008

Contents

1 Thrombin: Structure, Functions, and Regulation	1
Enrico Di Cera and Andras Gruber	
2 Thrombin: To PAR or Not to PAR, and the Regulation of Inflammation.....	19
Rithwik Ramachandran, Mahmoud El-Daly, Mahmoud Saifeddine, and Morley D. Hollenberg	
3 Regulation of Thrombin Receptor Signaling	47
JoAnn Trejo	
4 Thrombin and Activated Protein C: Integrated to Regulate Vascular Physiology	63
Matthias Riewald	
5 The Role of Thrombin in Vascular Development	81
Martin Moser and Cam Patterson	
6 The Role of Thrombin in Angiogenesis.....	93
Nikos E. Tsopanoglou and Michael E. Maragoudakis	
7 Thrombin and Thrombin Peptides in Wound Healing and Tissue Repair	115
Barbara Olszewska-Pazdrak, John S. Bergmann, Gerald M. Fuller, and Darrell H. Carney	
8 The Role of Thrombin and Thrombin Receptors in the Brain	133
Weibo Luo, Yingfei Wang, and Georg Reiser	
9 The Role of Thrombin in Tumor Biology	161
Boris Kobrin sky and Simon Karpatkin	

10 The Role of Thrombin and its Receptors in Epithelial Malignancies: Lessons from a Transgenic Mouse Model and Transcriptional Regulation.....	173
Zaidoun Salah, Sorina Grisaru-Granovsky, Myriam Maoz, Beatrice Uziely, Irit Cohen, Hagit Turm, Tamar Peretz, and Rachel Bar-Shavit	
11 Anti-thrombotic Therapy in Cancer Patients	189
Gloria A. Petralia and Ajay K. Kakkar	
12 Thrombin Receptor Modulators: Medicinal Chemistry, Biological Evaluation, and Clinical Application	205
Cailin Chen, Bruce E. Maryanoff, and Patricia Andrade-Gordon	
13 Novel Anticoagulant Therapy: Principle and Practice	237
Shaker A. Mousa	
Index.....	259

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Chapter 1

Thrombin: Structure, Functions, and Regulation

Enrico Di Cera and Andras Gruber

Abstract Thrombin is a Na^+ -activated, allosteric serine protease that plays opposing functional roles in blood coagulation. Binding of Na^+ is the major driving force behind the procoagulant, prothrombotic, and signaling functions of the enzyme, but is dispensable for cleavage of the anticoagulant protein C. The anticoagulant function of thrombin is under the allosteric control of the cofactor thrombomodulin. Recent structural advances have shed light on the remarkable molecular plasticity of this enzyme and the molecular underpinnings of thrombin allostery mediated by binding to exosite I and the Na^+ site. Thrombin exists in three forms – E^* , E, and $\text{E}:\text{Na}^+$, which interconvert under the influence of ligand binding to distinct domains. The transition between the Na^+ -free slow form E and the Na^+ -bound fast form $\text{E}:\text{Na}^+$ involves the structure of the enzyme as a whole, and so does the interconversion between the two Na^+ -free forms E^* and E. E^* is most likely an inactive form of thrombin, unable to interact with Na^+ and substrate.

1.1 Introduction

Thrombin is a serine protease of the chymotrypsin family, which includes enzymes involved in digestion and degradative processes, blood coagulation, cell-mediated immunity and cell death, complement, fibrinolysis, fertilization, and embryonic development. Once generated in the blood from its inactive precursor prothrombin, thrombin plays two important and paradoxically opposing functions (Di Cera 2008). It acts as a procoagulant factor when it converts fibrinogen into an insoluble fibrin clot that anchors platelets to the site of lesion and initiates processes of wound repair.

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This action is reinforced and amplified by activation of the transglutaminase factor XIII that covalently stabilizes the fibrin clot, the inhibition of fibrinolysis via activation of TAFI, and the proteolytic activation of factors V, VIII, and XI. In contrast, thrombin acts as an anticoagulant through activation of protein C. This function unfolds *in vivo* upon binding to thrombomodulin, a receptor on the membrane of endothelial cells. Binding of thrombomodulin suppresses the ability of thrombin to cleave fibrinogen and PAR₁, but enhances >1,000-fold the specificity of the enzyme toward the zymogen protein C. The reaction is further enhanced by the presence of a specific endothelial cell protein C receptor. Activated protein C (APC) cleaves and inactivates factors Va and VIIIa, two essential cofactors of coagulation factors Xa and IXa, which are required for thrombin generation, thereby downregulating both the amplification and progression of the coagulation cascade (Esmon 2003). Hijacking of thrombin by thrombomodulin and activation of protein C in the microcirculation constitute the natural anticoagulant pathway that prevents massive intravascular conversion of fibrinogen into an insoluble clot upon thrombin generation. In addition, thrombin is irreversibly inhibited at the active site by the serine protease inhibitor antithrombin with the assistance of heparin (Gettins 2002; Olson and Chuang 2002) and by the thrombin-specific heparin cofactor II (Tollefsen 2007). Important cellular effects are triggered by thrombin cleavage of protease-activated receptors (PARs) (Coughlin 2000), which are members of the G-protein-coupled receptor superfamily (Brass 2003). Four PARs have been identified, which share the same basic mechanism of activation: thrombin and other proteases cleave at a specific site within the extracellular N-terminus, exposing a new N-terminal tethered ligand domain that binds and activates the cleaved receptor (Coughlin 2000). Thrombin activation of PAR₁ (Vu et al. 1991), PAR₃ (Ishihara et al. 1997; Sambrano et al. 2001), and PAR₄ (Kahn et al. 1998; Xu et al. 1998; Nakanishi-Matsui et al. 2000) obeys this mechanism. PAR₁ is responsible for platelet activation in humans at low thrombin concentrations and its action is reinforced by PAR₄ at high enzyme concentrations (Coughlin 2000). Activation of PAR₁ and PAR₄ triggers platelet activation and aggregation and mediates the prothrombotic role of thrombin in the blood. PAR₃ is not present on human platelets, but is widely and abundantly expressed in other cell types. In the mouse, signaling in platelets is mediated entirely by PAR₄, with PAR₃ facilitating PAR₄ cleavage at low thrombin concentrations (Kahn et al. 1998; Nakanishi-Matsui et al. 2000). The efficiency of the coagulation cascade depends on the balance between the procoagulant and anticoagulant pathways. Thrombin is the key arbiter of this balance by virtue of its dual role and has therefore received utmost attention in structure–function studies and as a target of anticoagulant therapy (Bates and Weitz 2006).

1.2 Thrombin and Na⁺

The most striking feature of thrombin is its ability to interact with Na⁺ and the effect that Na⁺ binding carries on physiologically important interactions with procoagulant (fibrinogen), prothrombotic (PARs), and anticoagulant (protein C) substrates

(Dang et al. 1995, 1997; Di Cera et al. 2007). Wells and Di Cera (1992) demonstrated that the Na^+ activation of thrombin is specific and allosteric, as expected for a Na^+ -activated type II enzyme (Di Cera 2006). The property is shared with other clotting factors and proteases involved in immune response (Dang and Di Cera 1996; Krem and Di Cera 2001; Di Cera 2006). Na^+ binding converts thrombin from a low-activity slow (Na^+ -free) form to a high-activity fast (Na^+ -bound) form (Wells and Di Cera 1992). The slow and fast forms are significantly (2:3 ratio) populated under physiologic conditions because the K_d for Na^+ binding is 110 mM at 37°C (Wells and Di Cera 1992; Prasad et al. 2003; Bah et al. 2006) and the physiologic $[\text{NaCl}]$ (140 mM) is not sufficient for saturation. Hence, the slow–fast equilibrium in vivo is optimally poised for allosteric regulation, and this is all the more significant in view of the fact that the procoagulant and anticoagulant activities of thrombin are partitioned between the fast and slow forms, respectively (Dang et al. 1995). Na^+ binding is required for optimal cleavage of fibrinogen and activation of factors V, VIII, and XI necessary for the explosive generation of thrombin in the coagulation cascade, but is dispensable for cleavage of protein C. This proves that Na^+ is the major driving force behind the procoagulant role of the enzyme in the blood (Di Cera et al. 2007; Di Cera 2008). Na^+ binding also promotes the prothrombotic and signaling functions of the enzyme by enhancing the cleavage of PAR1, PAR3, and PAR4. Owing to the allosteric nature of thrombin, any effect that destabilizes Na^+ binding stabilizes the slow form and produces an anticoagulant effect by prolonging the clotting time (reduced fibrinogen cleavage) and reducing platelet activation (reduced PAR1 cleavage). Indeed, several naturally occurring mutations of the prothrombin gene, such as prothrombin Frankfurt, Salakta, Greenville, Scranton, Copenhagen, and Saint Denis, affect residues linked to Na^+ binding (Pineda et al. 2004a) and are often associated with bleeding.

1.3 Thrombin Structure

Thrombin bears the chymotrypsin-like fold where two 6-stranded β -barrels come together asymmetrically to host at their interface the residues of the catalytic triad, H57, D102, and S195 (chymotrypsinogen numbering). Thrombin is composed of two polypeptide chains of 36 (A chain) and 259 (B chain) residues that are covalently linked through a disulfide bond between residues C1 and C122 (Fig. 1.1). The standard “Bode” orientation puts the A chain in the back of the molecule, opposite to the front hemisphere of the B chain that hosts the entrance to the active site and all known functional epitopes of the enzyme (Pineda et al. 2004a). The A chain has received little attention in thrombin studies and is considered an appendage of the activation process from prothrombin. However, several naturally occurring mutations of prothrombin involve residues of the A chain and are associated with severe bleeding. The functional defects in prothrombins Denver (E8K and E14cK), Segovia (G14mR), and San Antonio (R15H) have been attributed to perturbation of the zymogen $\rightarrow$ enzyme conversion and processing by factor Xa, resulting in severe

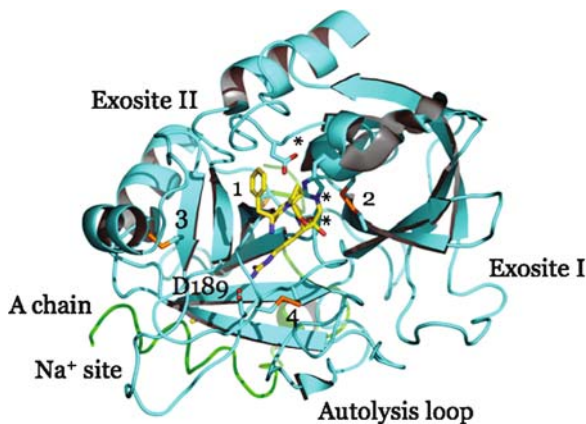


Fig. 1.1 Structure of thrombin bound to the active site inhibitor PPACK (stick model) and Na^+ (ball). The A chain runs in the back of the B chain. Disulfide bonds are numbered 1 (C1–C122), 2 (C48–C52), 3 (C168–C182), 4 (C191–C220). Relevant domains are noted. Catalytic residues (H57, D102, S195) are marked by asterisk, and D189 is labeled. The bound Na^+ is nestled between the 220-loop and the 186-loop and is within 5 Å from the side chain of D189. Numbering refers to chymotrypsin(ogen) (see *Color Plates*)

bleeding. Such explanation is obvious for the G14mR and R15H mutations that affect the P1 (R15) and P2 (G14m) sites of recognition by factor Xa, but not for the E8K and E14cK mutations of prothrombins Denver. Other naturally mutations, such as deletion of K9 or K10, are also associated with severe bleeding. Interestingly, the defect causes impaired fibrinogen and PAR_1 cleavage, reduced response to Na^+ activation and long-range perturbation of active site residues (De Cristofaro et al. 2006). The A chain is rich in charged residues that make polar interactions with partners of the B chain. Importantly, a significant number of negatively charged residues cluster toward the C-terminus, in close proximity to the Na^+ site. These charged residues may influence Na^+ binding or allosteric transduction, or both. The A chain is also optimally shaped to provide communication between the Na^+ site and the back of the active site region and could therefore influence substrate recognition and catalysis.

Trypsin-like specificity for Arg residues at P1 is conferred to thrombin by the presence of D189 in the S1 site occupying the bottom of the catalytic pocket. Thrombin has a preference for small and hydrophobic side chains at P2 that pack tightly against the hydrophobic wall of the S2 site defined by residues Y60a–P60b–P60c–W60d of the 60-loop. Residues at P3 point away from the thrombin surface, whereas aromatic and hydrophobic residues at P4 tend to fold back on the thrombin surface and engage the aryl binding site defined by L99, I174, and W215. The autolysis loop shapes the lower rim of access to the active site and contributes to the recognition of fibrinogen. The loop centered on K70 defines exosite I and is homologous to the Ca^{2+} -binding loop of trypsin and chymotrypsin. Exosite I contains several positively charged residues that give rise to an intense electrostatic field. The field

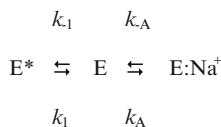
provides steering and optimal pre-orientation for fibrinogen, thrombomodulin, the natural inhibitor hirudin and PAR₁ to facilitate the formation of a productive complex upon binding. Structural and site-directed mutagenesis data support exosite I as a binding epitope for fibrinogen, fibrin, thrombomodulin, and the thrombin receptors PAR₁ and PAR₃ (Di Cera et al. 2007; Di Cera 2008). On the side of the enzyme opposite to exosite I, a C-terminal helix and its neighbor domains host a number of positively charged residues and define exosite II. This site is the locale for interaction with polyanionic ligands such as heparin and glucosaminoglycans. Heparin enhances inhibition of thrombin by antithrombin via a template mechanism in which a high-affinity heparin–antithrombin complex is first formed and then docked into exosite II and the thrombin active site by electrostatic coupling (Gettins 2002; Olson and Chuang 2002; Dementiev et al. 2004; Li et al. 2004). Exosite II is also the locale for thrombin interaction with the platelet receptor GpIb (De Cristofaro et al. 2000; Ramakrishnan et al. 2001; Celikel et al. 2003; Dumas et al. 2003).

The first X-ray structure of thrombin was solved in 1992 and revealed relevant information on the overall fold of the enzyme and especially on the arrangement of loops involved in macromolecular substrate recognition (Bode et al. 1992). The Na⁺ binding site of thrombin was first identified crystallographically in 1995 from Rb⁺ replacement (Di Cera et al. 1995). Na⁺ binds 16–20 Å away from residues of the catalytic triad and within 5 Å from D189 in the S1 site, nestled between the 220- and 186-loops and coordinated octahedrally by two carbonyl O atoms from the protein (residues R221a and K224) and four buried water molecules. Structural biology has revealed important information on how thrombin utilizes both the active site and exosites for interaction with substrates, inhibitors, and effectors. Information on how thrombin recognizes substrate at the active site has come from the structure of the enzyme in complex with the irreversible active site inhibitor H-D-Phe-Pro-Arg-CH₂Cl (PPACK) (Bode et al. 1992). Arg at P1 ion-pairs to D189 in the S1 site; Pro at P2 fits snugly against P60b, P60c, and W60d in the S2 site; and Phe at P3, in the d-enantiomer, makes an edge-to-face interaction with W215 in the aryl binding site. The PPACK-inhibited structure reveals interactions that are relevant to recognition of natural substrates and confirms the key role played by the H-bonding network found within the active site of all trypsin-like enzymes bound to substrate (Hedstrom 2002). Crucial components of this network are the bidentate ion-pair between D189 and the guanidinium group of Arg at P1, the H-bonds of the carbonyl O atom of the P1 residue with the N atoms of G193 and S195 forming the oxyanion hole, the H-bond between the N atom of the P1 residue and the carbonyl O atom of S214, and the H-bonds between the backbone O and N atoms of the P3 residue with the N and O atoms of G216. This important arrangement of H-bonds has been documented in the structures of thrombin bound to fragments of the natural substrates fibrinogen (Stubbs et al. 1992), PAR4 (Bah et al. 2007), and factor XIII (Sadasivan and Yee 2000). The structure of thrombin in complex with the potent natural inhibitor hirudin has revealed how thrombin recognizes ligands at exosite I (Rydel et al. 1991). Hirudin blocks access to the active site of thrombin using its compact N-terminal domain and binds to exosite I via its extended, acidic C-terminal domain. The mode of interaction of the C-terminal domain of hirudin has later been documented in the

structures of thrombin bound to hirugen (Vijayalakshmi et al. 1994), fibrinogen (Pechik et al. 2004, 2006), PAR₁ (Mathews et al. 1994; Gandhi et al. 2008), PAR₃ (Bah et al. 2007), thrombomodulin (Fuentes-Prior et al. 2000), and heparin cofactor II (Baglin et al. 2002). Finally, the role of exosite II has been documented eloquently in the structures of thrombin bound to heparin (Carter et al. 2005), the fibrinogen γ' peptide (Pineda et al. 2007), and GpIb (Celikel et al. 2003; Dumas et al. 2003).

1.4 Kinetics of Na⁺ Activation

The discovery of the Na⁺ effect on thrombin has provided a coherent framework to understand the structure and function of the enzyme, rationalized the molecular origin of the defects associated with several naturally occurring mutations of the prothrombin gene and offered an effective strategy to engineer thrombin for optimal anticoagulant activity in vivo which may one day translate into new therapeutic tools. Na⁺ binding to thrombin is linked to a significant increase in intrinsic fluorescence with an initial rapid phase (in the microsecond time scale) that cannot be resolved within the dead time (<0.5 ms) of the spectrometer, followed by a single exponential slow phase (in the microsecond time scale) with a k_{obs} that decreases as [Na⁺] increases (Bah et al. 2007). Scheme 1 shows the two-step mechanism of Na⁺ binding to thrombin.



Scheme 1 The two-step mechanism of Na⁺ binding to thrombin

Thrombin exists in equilibrium between two forms, E* and E, which interconvert with kinetic rate constants k_1 and k_{-1} . Of these forms, only E can interact with Na⁺ with a rate constant k_A to populate E:Na⁺, which may dissociate into the parent components with a rate constant k_{-A} . The fast phase is due to the E–E:Na⁺ interconversion involving Na⁺ binding/dissociation, and the slow phase is due to the E–E* interconversion that precedes Na⁺ binding. The three species in Scheme 1 portray thrombin in the Na⁺-free (E and E*) and Na⁺-bound (E:Na⁺) forms. E and E:Na⁺ in Scheme 1 are the two active forms of thrombin that account for the dependence of k_{cat} on [Na⁺] and correspond to the slow (E) and fast (E:Na⁺) forms originally defined by Wells and Di Cera (1992). E* is a form of thrombin that cannot interact with Na⁺ and substrate.

Precise determinations of the value of K_A as a function of temperature enable dissection of the thermodynamic components of Na⁺ binding (Bah et al. 2006). Binding of Na⁺ is characterized by a large enthalpy change of $-22 \text{ kcal mol}^{-1}$ that is compensated by a large entropy loss of $-64 \text{ cal mol}^{-1}\text{K}^{-1}$. As a result of the enthalpy–entropy energetic compensation, the binding affinity of Na⁺ is relatively weak (K_d in the millimolar range), as seen for many other M⁺-activated enzymes.

An important consequence of the large enthalpy change is that the value of K_A becomes only about 10 M^{-1} at 37°C , which implies that under physiologic conditions of temperature and $[\text{NaCl}] = 140 \text{ mM}$, thrombin is only 60% bound to Na^+ . The fraction of thrombin in the procoagulant $\text{E}:\text{Na}^+$ form is about 60%, and the anticoagulant form E accounts for about 40%. The temperature dependence also demonstrates that, of the two Na^+ -free forms, E^* represents $<1\%$ of the population of thrombin molecules at 37°C . Thrombin has a total of nine Trp residues located in the B chain anywhere from 13 to $35 \text{ \AA}$ from the bound Na^+ (Pineda et al. 2004a). Single-site Phe mutants of each of the nine Trp residues were used to identify fluorophores responsible for the spectral changes associated with Na^+ binding (Bah et al. 2006). The 10% total increase in fluorescence observed for wild type is retained by five Trp mutants, namely, W29F, W51F, W60dF, W96F, and W237F. Two mutants of thrombin, W148F and W207F, experience $>30\%$ loss in total fluorescence change upon Na^+ binding. On the other hand, W141F and W215F lose $>70\%$ of the total fluorescence change. The fast phase of fluorescence increase directly linked to the transition from E to $\text{E}:\text{Na}^+$ in Scheme 1 is affected in all Phe mutants, vouching for a global effect of Na^+ binding on thrombin structure. W141 and W215 make a large contribution to the fluorescence change induced by Na^+ binding, and their mutation to Phe abrogates the fast phase completely. This implies that the environments of W141 and W215 change in the $\text{E}^* \rightarrow \text{E}$ conversion, and more drastically in the conversion of $\text{E} \rightarrow \text{E}:\text{Na}^+$.

1.5 Structures of E^* , E, and $\text{E}:\text{Na}^+$

Current structural information on the molecular basis of Na^+ -dependent allostery accounts for many important functional differences between the E and $\text{E}:\text{Na}^+$ forms. However, the documented structural changes are limited and do not explain the full complexity of the allosteric transition captured by functional studies that vouch for a remarkable conformational transition that transduces Na^+ binding into a global, long-range perturbation of the enzyme. With this caveat in mind, we will now analyze the results of recent crystallographic studies of the three forms of thrombin – E^* , E, and $\text{E}:\text{Na}^+$.

Structures of E and $\text{E}:\text{Na}^+$ are highly similar, with rms deviations of the C α traces of only $0.38 \text{ \AA}$. There are five main structural differences between the slow (E) and fast ($\text{E}:\text{Na}^+$) forms of thrombin: (1) the R187–D222 ion-pair, (2) orientation of D189 in the primary specificity pocket, (3) conformation of E192 at the entrance of the active site, (4) position of the catalytic S195, and (5) architecture of the water network spanning $>20 \text{ \AA}$ from the Na^+ site to the active site. Much of the activating effect of Na^+ can be explained in terms of the more favorable orientation of D189 in the S1 site, that improves K_m , and of S195 in the active site, that improves k_{cat} . The most significant structural change between the slow and fast forms provides evidence of long-range communication between the Na^+ site and the active site. In the fast form, there is a network of 11 water molecules that connect through

H-bonds the bound Na^+ to the O_γ atom of S195, located >15 Å away. The network provides the long-range connectivity needed to allosterically communicate information from the Na^+ site to the active site S195 and to residues involved in substrate recognition, such as D189 and E192. In the slow form, only seven water molecules occupy positions in the network equivalent to those seen in the fast form and the connectivity is radically altered.

The available structures of the slow form E and fast form $\text{E}:\text{Na}^+$ account for some basic functional properties of the two allosteric conformations of thrombin and explain most of the kinetic features linked to Na^+ binding and allosteric activation. However, rapid kinetic measurements of Na^+ binding clearly demonstrate that E is in equilibrium with a third thrombin form, E^* , that is unable to interact with Na^+ (Scheme 1). The structural nature of E^* is of considerable interest. E^* has been suggested to portray an “inactive” slow form, unable to bind Na^+ and substrate or inhibitors at the active site. That hypothesis has gained prominence recently in the context of structure of the thrombin mutant D102N. The overall fold of D102N is similar to that in wild type, with a backbone rms deviation of 0.77 Å, compared to the Na^+ -bound fast form (Pineda et al. 2004a). The structure shows changes in the primary specificity pocket and in the Na^+ binding site that are unprecedented in thrombin and the entire realm of serine proteases. These changes provide a plausible interpretation of the peculiar properties of the E^* form. The 215–219 β -strand collapses into the primary specificity pocket. The side chain of R221a, located >10 Å away from the site of mutation, rotates 95° and brings the guanidinium group in contact with D189 in the primary specificity pocket. Altogether, the drastic shifts of W215 and R221a produce a conformation of thrombin that is self-inhibited by the hydrophobic engagement of the 60-loop and active site H57 by Trp-215, and of the acidic moiety of D189 in the primary specificity pocket by R221a. It should be pointed out, however, that although this structure reveals drastic changes at the level of W215 and perturbation of W141, it fails to account for changes at other Trp residues that are detected by fluorescence measurements. The true extent of conformational perturbation accompanying Na^+ binding to thrombin may ultimately defy resolution by X-ray structural biology.

1.6 Thrombin Interaction with Protein C

Two well-documented pathways of allosteric regulation exist in thrombin: one involves the Na^+ site and the other involves exosite I. As we have seen, binding of Na^+ to thrombin enhances activity toward procoagulant and prothrombotic substrates such as fibrinogen and PARs, whereas binding of thrombomodulin to exosite I enhances activity toward the anticoagulant protein C. Thrombin is the only enzyme in the blood capable of activating protein C (Di Cera 2003). Activated protein C is a natural anticoagulant that inactivates factors Va and VIIIa with the assistance of protein S, thereby promoting the downregulation of the coagulation cascade. The interaction of thrombin with protein C has been studied in considerable

detail. Under physiologic concentrations of Ca^{2+} , thrombin has only marginal affinity for protein C in the absence of thrombomodulin. The presence of thrombomodulin increases the $k_{\text{cat}}/K_{\text{m}}$ of thrombin for protein C >1,000-fold because of a tenfold decrease in K_{m} and a 100-fold increase in k_{cat} (Esmon et al. 1983). In contrast to such drastic functional effects, the structure of thrombin bound to a fragment of thrombomodulin at exosite I failed to reveal significant conformational changes in the active site or other regions of thrombin (Fuentes-Prior et al. 2000). Such changes might have been obliterated by the presence of the active site inhibitor used in the crystallization. A number of peptides targeting exosite I influence allosterically the active site of thrombin, bring about significant changes in activity and even substrate specificity. One of these peptides, hirugen, is derived from the C-terminal fragment of hirudin. The structure of thrombin bound to hirugen was solved with the active site free (Vijayalakshmi et al. 1994), but again failed to reveal any significant conformational changes as for the thrombomodulin-bound structure (Fuentes-Prior et al. 2000). Solution to this conundrum has come unexpectedly from studies of how thrombin recognizes the receptors PAR_1 , PAR_3 , and PAR_4 (Bah et al. 2007; Gandhi et al. 2008).

1.7 Thrombin Interaction with the PARs

The prothrombotic role of thrombin depends mainly on cleavage of protease-activated receptors (PARs), which are members of the G-protein-coupled receptor superfamily (Coughlin 2000; Brass 2003). The potent natural inhibitor hirudin targets exosite I via its extended, acidic C-terminal domain (Rydel et al. 1991). An hirudin-like acidic domain is present in PAR_1 and PAR_3 immediately downstream of the tethered ligand domain and has been invoked in the ability of these receptors to engage exosite I of thrombin (Vu et al. 1991; Kahn et al. 1998). Recognition of PAR_4 is less dependent on exosite binding and relies almost entirely on the active site moiety (Ayala et al. 2001), especially through a pair of Pro residues at the P2 and P4 positions (Cleary et al. 2002; Jacques and Kuliopulos 2003). In addition, PAR_4 constructs carrying an hirudin-like acidic domain fail to produce enhanced binding or catalytic processing (Jacques and Kuliopulos 2003), suggesting that PAR_4 folds into a conformation unable to bind to exosite I. The cofactor function of PAR_3 on PAR_4 cleavage and activation by thrombin in murine platelets (Kahn et al. 1998; Nakanishi-Matsui et al. 2000) implies binding of both PAR_4 and the cleaved form of PAR_3 to thrombin to form a ternary complex. After cleavage of PAR_3 , thrombin would remain bound to the receptor via exosite I. This complex would then engage PAR_4 for proteolytic activation optimized by an allosteric effect of PAR_3 bound to exosite I on the active site moiety. A similar mechanism likely exists in human platelets, where PAR_4 can be cofactored by other receptors (Kahn et al. 1998). The cofactor function of PAR_3 on PAR_4 cleavage and activation has many features in common with the interaction of thrombin with protein C that is cofactored by thrombomodulin. Structural studies of thrombin–PAR interactions should reveal important details on the allosteric mechanism underlying binding to exosite I.

Murine thrombin inactivated with the single site mutation S195A was crystallized in complex with the extracellular fragment of murine PAR₃ (Bah et al. 2007), ³⁸SFNGGPQNTFEEFPLS-DIE⁵⁶, corresponding to the sequence downstream of the cleavage site at K37 and containing the hirudin-like motif ⁴⁷FEEFP⁵¹ predicted to bind to exosite I (Nakanishi-Matsui et al. 2000; Jacques and Kuliopulos 2003). The PAR₃ fragment engages exosite I of murine thrombin in a number of well-defined interactions. Overall, the surface of interaction between thrombin and PAR₃ is composed of a hydrophobic patch surrounded by a periphery of polar/electrostatic contacts. The presence of PAR₃ bound to exosite I causes a rearrangement of the 60-loop lining the upper rim of the active site entrance. In the free enzyme the indole ring of W60d partially occludes access to the active site and restricts specificity toward physiologic substrates and inhibitors. When PAR₃ binds to exosite I, the 60-loop shifts 3.8 Å upward and causes a 180° flip of W60d around the Cβ–Cγ bond that projects the indole ring into the solvent and opens up the active site fully. Key to this allosteric effect is the ability of W60d to move like a flap and regulate substrate diffusion into the active site. The structural flexibility documented for this and other Trp residues of thrombin is in agreement with recent spectroscopic measurements (Bah et al. 2006).

The murine thrombin mutant S195A was also crystallized in complex with the extracellular fragment of murine PAR₄ (Bah et al. 2007), ⁵¹KSSDKPNPR↓GYPGKFCANDSDTL-ELPASSQA⁸¹ (↓ = site of cleavage), containing the cleavage site at R59. Overall, the thrombin conformation is similar to that seen in the thrombin–PAR₃ complex (rmsd, 0.52 Å), but W60d retains its canonical orientation with the indole ring pointing down over the active site in contact with PAR₄. Of great importance are the contacts made by PAR₄ with the active site of thrombin. R59 penetrates the primary specificity pocket and engages several residues. Together, P56 and P58 at the P4 and P2 positions of substrate produce a clamp that latches PAR₄ onto the active site of thrombin utilizing a large hydrophobic surface from the aryl binding site on one side and the 60-loop on the opposite side of the catalytic H57. The conformation of the ⁵⁶PNPR⁵⁹ sequence of PAR₄ is practically identical to that predicted by NMR studies of the human PAR₄ sequence ⁴⁴PAPR⁴⁷ (Cleary et al. 2002) and supports the critical role of this Pro pair in thrombin recognition uncovered by functional studies (Jacques and Kuliopulos 2003). Downstream from the scissile bond, the P1' residue G60 initiates a turn followed by a short helical segment stabilized by a H-bond between the O atom of P62 and the N atom of C66. Without P62, the fragment of PAR₄ would likely pursue the 30-loop and exosite I of thrombin. The helix turn stabilizes and redirects PAR₄ toward the autolysis loop right below the entrance to the active site. The mode of interaction of PAR₄ with thrombin calls for numerous interactions with the active site and the aryl binding site, leaving exosite I and the 30-loop free. Cleavage of PAR₄ does not require binding to exosite I of thrombin, in agreement with the findings of earlier mutagenesis studies (Nakanishi-Matsui et al. 2000; Jacques and Kuliopulos 2003). The strategy used by PAR₄ to contact the active site of thrombin in a way that avoids interaction with exosite I is highly relevant to the interaction of thrombin with protein C, for which no structural information is currently avail-

able. The obvious conclusion to be drawn from the structures of murine thrombin bound to PAR₃ and PAR₄ is that the cleaved form of PAR₃ bound to exosite I does not interfere with the binding of the intact PAR₄ to thrombin. The cleaved PAR₃ and intact PAR₄ fragments bind to thrombin without overlap and can generate a ternary complex where PAR₃ functions as a cofactor that allosterically optimizes PAR₄ cleavage (Fig. 1.2).

The structure of the thrombin–PAR₃ complex offers convincing evidence that the position of W60d and the 60-loop can be modified allosterically by binding to exosite I when the active site of thrombin is free. A recent structure of thrombin bound to PAR₁ provides crystallographic evidence of a much larger conformational change experienced by thrombin when exosite I is bound to a ligand (Gandhi et al. 2008). Human thrombin inactivated with the single site mutation D102N was crystallized in complex with the extracellular fragment of human PAR₁, ⁴²SFLLRNPNDKYEPFWEDEEKN⁶², corresponding to the sequence downstream from the cleavage site at R41 and containing the hirudin-like motif ⁵²YEPFWE⁵⁷ predicted to bind to exosite I (Vu et al. 1991; Coughlin 2000). The structure documents a large conformational change that propagates from F34 and R73 in exosite I, to W215 in the aryl binding site and R221a in the 220-loop located up to 28 Å away on the opposite side of the active site relative to exosite I. The peculiar self-inhibited E* conformation documented originally for D102N (Pineda et al. 2006) can therefore convert into a catalytically active conformation through a structural transition that can be traced to a set of residues organized in four layers. A first layer directly in contact with ligands recognizing exosite I (F34 and R73), a second

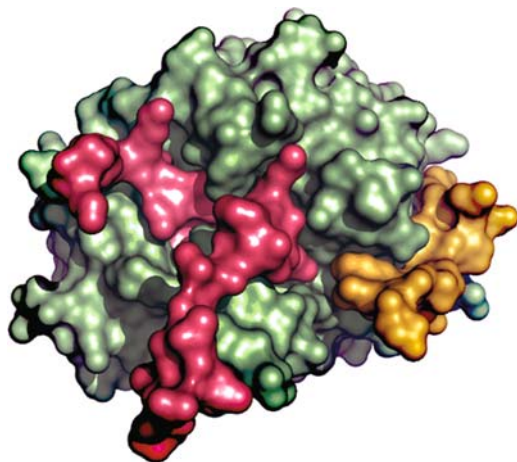


Fig. 1.2 Putative ternary complex of thrombin, cleaved PAR₃, and PAR₄ derived from an overlay of the crystal structures of the murine thrombin–PAR₄ and thrombin–PAR₃ complexes. Thrombin refers to the thrombin–PAR₄ complex. Binding of cleaved PAR₃ to exosite I does not interfere with binding of PAR₄ to the active site of the enzyme. Cleaved PAR₃ may therefore act as a cofactor of PAR₄ cleavage by thrombin (*see Color Plates*)

layer of “transducing” residues connecting to the 141–146 β -strand (M32 and Q151), a third layer comprising the interactions between the 141–146 and 191–193 β -strands (W141, N143, and E192), and a final layer where such interactions are transmitted to the 215–219 β -strand and the 220-loop via the C191:C220 disulfide bond and E146.

1.8 Dissociating Procoagulant and Anticoagulant Activities

The multifunctional nature of thrombin has long motivated interest in dissociating its procoagulant and anticoagulant activities. Thrombin mutants with anticoagulant activity help rationalize the phenotypes of several naturally occurring mutations and could eventually provide new tools for pharmacological intervention (Bates and Weitz 2006). A comprehensive library of Ala mutants was recently utilized to map the epitopes recognizing fibrinogen, protein C, and thrombomodulin in order to identify residues that contribute differentially to the procoagulant and anticoagulant functions of thrombin. Residues important for fibrinogen recognition are distributed over the entire surface of contact between enzyme and substrate and involve the 60-loop, exosite I, the primary specificity pocket, the aryl binding site, and the Na^+ binding site (Di Cera et al. 2007; Di Cera 2008). The surface of recognition between thrombin and protein C changes significantly upon thrombomodulin binding and is reduced mainly to the primary specificity pocket and portions of the 60-loop in the presence of cofactor. This makes it possible to identify residues that are significantly more important for fibrinogen recognition than protein C activation and to engineer thrombin mutants with enhanced anticoagulant activity in three steps: (1) identify residues that contribute differentially to fibrinogen and protein C recognition, (2) select among these residues those that make independent contributions to substrate recognition, and (3) construct multiple mutations involving these residues to achieve additivity. Targets can be set for optimal anticoagulant activity in vivo by demanding safety and potency of the constructs. For a mutant to be safe, activity toward fibrinogen should be $<0.1\%$ of that of wild type. Potency demands activity toward protein C to be $>10\%$ of that of wild type. These targets define a region in Fig. 1.3 where “ideal” anticoagulant thrombin mutants should map for selection. None of the 80 Ala mutants reported in Fig. 1.3 map in that region, but W215A and E217A stand out. Because W215 and E217 participate in fibrinogen recognition via distinct interactions, the double mutant W215A/E217A (WE) was constructed (Cantwell and Di Cera 2000) hoping for additivity of mutational effects that would boost the anticoagulant activity relative to the single mutations. Additivity was indeed found and WE mapped in the desired section in Fig. 1.3 (Cantwell and Di Cera 2000). The crystal structure of WE in the absence of inhibitors shows a conformation with the active site occluded by a collapse of the 215–217 strand, whereas the PPACK-bound form is similar to that of wild type (Pineda et al. 2004b). The mutant is practically inactive toward synthetic and natural substrates, and recovers activity only in the presence of thrombomodulin and protein

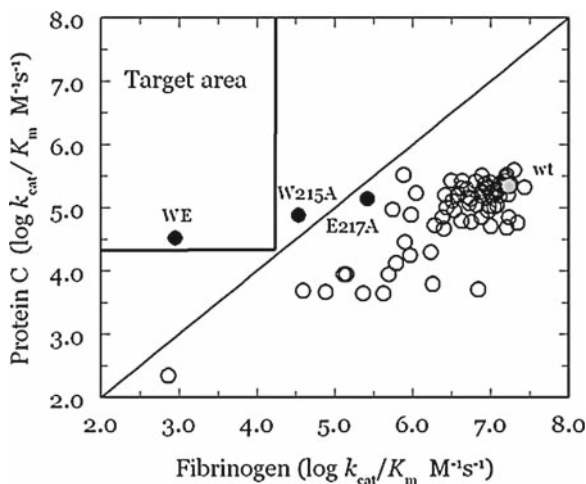


Fig. 1.3 Specificity constants k_{cat}/K_m for the hydrolysis of fibrinogen and protein C, in the presence of 10 mM thrombomodulin and 5 mM CaCl_2 , under experimental conditions of 5 mM Tris, 0.1% PEG, 145 mM NaCl, pH 7.4, at 37°C. Plotted are the wild-type (gray) and 80 Ala mutants of thrombin. Safety and potency of an anticoagulant thrombin mutant for in vivo applications demand $<0.1\%$ activity toward fibrinogen and $>10\%$ activity toward protein C compared to wild type. These boundaries define a target region in the plot where mutations should map. W215A and E217A are the most promising single Ala mutants. Combination of the two mutations in the W215A/E217A produces additivity and a construct with the required anticoagulant profile

C. Hence, WE is presumably stabilized in the E^* form and converts to the E form upon binding of thrombomodulin following a molecular transition similar to that documented for the interaction of the D102N mutant with PAR_1 . These are the most desirable properties for a recombinant thrombin to be used in vivo as an anticoagulant.

1.9 WE: A Prototypic Anticoagulant/Antithrombotic Thrombin

Systemic administration of activated protein C (APC) has antithrombotic and anti-inflammatory effects that are now utilized in the treatment of severe sepsis (Bernard et al. 2001). Since infused thrombin activates protein C and APC is antithrombotic, thrombin infusion could act, in theory, as an antithrombotic agent. The thrombin analog WE was tested in a wide dose range for safety and efficacy in a baboon model of acute vascular graft thrombosis (Gruber et al. 2002, 2007). In this model, a permanent, surgically implanted arteriovenous shunt is temporarily extended with a thrombogenic vascular graft segment. Acute thrombus formation is visualized and quantified in real time using gamma camera imaging the deposition of radiolabeled platelets in the graft. WE was initially administered as an intravenous bolus in order

to evaluate pharmacokinetics. The lowest bolus dose of WE tested, $11 \mu\text{g kg}^{-1}$, reduced platelet accumulation by 80% 1 h after the beginning of thrombosis, and was at least as effective as the direct administration of 40-fold more (0.45 mg bolus/kg) APC. Baboons treated with WE at doses as high as $200 \mu\text{g kg}^{-1}$ showed no clinical or laboratory signs of thrombosis, hemorrhage, or organ failure. No procoagulant activity could be detected for up to 1 week in baboon plasma obtained following bolus WE administration. Meanwhile, rapid systemic anticoagulation was observed, which dissipated with the biological half life of circulating APC, as determined in previous experiments in baboons. Higher doses of WE ($>20 \mu\text{g kg}^{-1}$) had pronounced anticoagulant effect, triggered by a burst in the levels of circulating endogenous APC following injection of WE (Gruber et al. 2006). High-dose WE infusion exhausted the cofactors of protein C activation before consumption of the substrate reserve and the process was rapidly downregulated $>90\%$ following a WE overdose. The exact molecular or cellular mechanism of this self-limiting pharmacological process is not yet known, but failure of protein C activation upon WE overdose can be overcome by cofactor supplementation using soluble recombinant thrombomodulin (Gruber et al. 2006). These results suggest that the thrombin–thrombomodulin complex is efficiently and rapidly inhibited *in vivo* and that thrombomodulin does not recycle rapidly once WE (and possibly thrombin) is bound to it. The true significance of this surprising finding is that pharmacological protein C activation by WE appears to be intrinsically safe, compared to all other accepted methods of anticoagulation, that are ultimately fatal when overdosed. Studies with low-dose WE infusions revealed that pharmacological levels of APC and marginal systemic anticoagulation could be maintained by continuous WE infusion for at least 5 h without substantial decrease in protein C levels. Since WE has potent antithrombotic effects even at very low doses that do not induce systemic anticoagulation (Gruber et al. 2007), natural protein C reserves and production could keep up with the consumption of protein C during sustained antithrombotic WE treatment.

The hemostatic safety of continuous WE infusion in comparison to equi-efficacious doses of low molecular weight heparin infusion for the prevention of acute vascular graft thrombus propagation in the baboon model was also evaluated. On the basis of previously established anticoagulant effect and antithrombotic efficacy of circulating APC in the baboon model, we originally predicted that, in systemic blood samples, an APTT prolongation of at least 1.5-fold over baseline, and APC levels at least 20-fold over baseline, sustained for at least 40 min, would result in a significant antithrombotic benefit. The results exceeded our expectations and we found that WE had potent antithrombotic effect even at a dose ($2.1 \mu\text{g kg}^{-1}$ per 70 min) when the APTT was not demonstrably affected (Gruber et al. 2007). This indicated that WE was a very potent antithrombotic agent in primates (Gruber et al. 2007). Low doses of WE (2.1 , 4.2 , or $8.3 \mu\text{g kg}^{-1}$ per 70 min) outperformed higher doses of exogenous APC (28 and $222 \mu\text{g kg}^{-1}$ per 70 min) and were as efficient as interventional doses of intravenous enoxaparin (325 – $2,600 \mu\text{g kg}^{-1}$ per 70 min) in preventing the propagation of thrombi. The lowest dose of WE tested still increased circulating APC levels by approximately fivefold (to about 20 ng mL^{-1}), and we do

not yet know whether even lower doses of WE that may not detectably increase APC levels would also be efficacious. The comparably effective plasma concentration of exogenous (recombinant) APC exceeded the endogenous APC level following low-dose WE infusion by severalfold. The fact that WE is very effective at such small doses strongly argues for limited receptor-mediated mechanism. The considerably higher antithrombotic efficacy of WE, compared to exogenous APC, suggests that APC generated by WE on the surface of endothelial cells may remain transiently bound to the receptor and elicit additional effects by signaling via PAR₁ (Feistritzer et al. 2006; Gruber et al. 2007). WE may be retained on the cell surface in the vicinity of PAR₁ by receptors other than thrombomodulin, similar to the case of its interaction with platelet GpIb which appears to be active only under shear flow conditions (Berny et al. 2008).

The molar ratio of the equi-efficacious doses of WE and enoxaparin exceeded 1:1,000. However, this extraordinary potency would be ultimately and clinically irrelevant if the safety profile of WE were not substantially different from other anticoagulants. The striking finding was that these potent antithrombotic doses of WE infusion did not impair primary hemostasis, an outcome never before seen in baboons that received comparably effective doses of commercially available antithrombotic agents (Gruber et al. 2007). Interestingly, the comparably antithrombotic, and reasonably low doses of WT thrombin, 40 $\mu\text{g kg}^{-1} \text{h}^{-1}$, and WE, about 2 $\mu\text{g kg}^{-1} \text{h}^{-1}$, appear to be at least one order of magnitude apart to the advantage of WE, despite the 90% reduced activity of WE toward protein C. One of the possible explanations for this discrepancy could be the propensity of wild-type thrombin to bind to and activate abundant prothrombotic substrates (e.g., fibrinogen, PAR₁) in the blood flow, leaving only a fraction of the dose to diffuse across the boundary layer of flow to surfaces. Meanwhile, binding and interaction of WE with fibrinogen in blood are markedly reduced, the rate of such interactions as fibrinogen cleavage and inhibition by antithrombin are delayed by several orders of magnitude. The delay leaves more time for the administered enzyme to reach surfaces and become available for interaction with or consumption by transmembrane cofactors and receptors, such as thrombomodulin and PAR₁. This can thus lead to effective activation of the protein-C-dependent pathways on solid surfaces such as the endothelium. Altogether, this makes WE thrombin an agent that targets thrombosis and helps explain the very high antithrombotic efficacy of this enzyme.

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Chapter 2

Thrombin: To PAR or Not to PAR, and the Regulation of Inflammation

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Abstract Thrombin, a key “final common pathway” coagulation cascade proteinase, can be envisioned as one of the body’s main “sentries,” always on the lookout to be rendered active at sites of injury or other stress inducers, and always ready to generate a variety of signals that trigger the defense responses that comprise the process termed *inflammation*. Thrombin does this job in a clever way, using mechanisms that range from the generation of fibrin from fibrinogen, to the activation of G-protein-coupled receptors. The novel way that thrombin acts on human platelets, by cleaving and stimulating proteolytically activated receptors (PARs), has defined a new role not only for thrombin but also for proteinases in general, as hormone-like agents. Thus, thrombin can be seen as a prototype for a number of proteinases that can regulate cell function either by unmasking the receptor-activating tethered ligand sequence of PARs or by silencing PARs by removing the “tethered ligand,” thereby preventing activation by other proteinases such as thrombin. To play its role in inflammation, thrombin acts not only via the PARs but also by other mechanisms, such as the activation of metalloproteinases, the generation of active peptides from fibrin and by using non-catalytic mechanisms to trigger cell signalling. This chapter summarizes the several mechanisms (both PAR and non-PAR-related) that thrombin can use to regulate cell and tissue function, with a particular focus on the inflammatory response.

2.1 Introduction

Thrombin is a clever enzyme. It acts only upon demand, and then uses a number of novel independent mechanisms to signal to cells and tissues in its vicinity. Further, in the course of the coagulation cascade, thrombin can switch its substrate specificity,

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upon binding thrombomodulin. It is thus not surprising that amongst serine proteinases, thrombin is very likely unique in terms of its diversity of functions. Originally discovered as a result of the hypothesis that the conversion of fibrinogen to fibrin was an enzymatic process catalysed by “fibrinferment,” (subsequently named *thrombin*; Schmidt 1872, 1892), thrombin’s actions for some time were thought to be involved principally in blood coagulation. However, observations of the ability of thrombin to activate platelets (Shermer et al. 1961; Schmid et al. 1962; Conley 1967), to stimulate mitogenesis (Chen and Buchanan 1975; Carney et al. 1978; Perdue et al. 1981), to affect nerve cell function (Gurwitz and Cunningham 1988) and to regulate vascular function (De Mey et al. 1982; Haver and Namm 1984; Walz et al. 1985) indicated that thrombin plays a much wider physiological role than was first anticipated. Given the many actions of thrombin, a search was soon on for the “receptor” that could explain its cellular actions, apart from its ability to cleave fibrinogen. This chapter, in concert with other chapters in this book, aims to outline the discovery of the cell surface targets for thrombin action and to summarize a number of thrombin’s effects that can be attributed to the activation of receptors for thrombin. One main focus will be on the role that these actions have in regulating an innate immune inflammatory response. As will be seen, a large number of the actions of thrombin are due to its regulation of the G-protein-coupled, proteolytically activated receptors (PARs) 1 and 4. But, PARs do not account for all of thrombin’s cellular actions. Thus, the chapter will also outline the cellular actions of thrombin that are regulated by mechanisms apart from the PARs, with a focus on inflammatory processes.

2.2 Thrombin and the Search for Its Receptor

The initial attempt to identify the receptor for thrombin used a ligand-binding approach with radiolabelled thrombin, which interacts with a high-affinity site on fibroblasts (Carney and Cunningham 1978; Van Obberghen-Schilling and Pouyssegur 1985). As outlined in more detail in the chapter in this volume by Olszewska-Pazdrak et al., binding competition studies with thrombin-derived peptide sequences identified a 23mer that could compete for thrombin binding and complement the mitogenic activity of proteolytically active thrombin. But, unlike thrombin, the peptide did not promote cell replication on its own, as does thrombin. A number of species have been detected upon gel electrophoresis by thrombin cross-linking methods, one of which is protease nexin, with a size of about 35 kDa. However, the high-affinity thrombin-binding constituent of about 150 kDa, that can be chemically cross-linked to bound thrombin, cannot account for thrombin’s mitogenic activity (Van Obberghen-Schilling and Pouyssegur 1985) and is yet to be identified.

A continued search was therefore on in the late 1980s to identify the thrombin receptor on human platelets and hamster lung fibroblasts that triggers platelet aggregation and the activation of fibroblast mitogenesis. It was an expression cloning

approach rather than the ligand-binding method that succeeded. Surprisingly, the result led to the cloning of a member of the G-protein-coupled receptor superfamily (Rasmussen et al. 1991; Vu et al. 1991; Coughlin 2000; Macfarlane et al. 2001; Hollenberg and Compton 2002; Ossovskaya and Bunnett 2004; Steinhoff et al. 2005). At that time, it was known that the mitogenic activity of thrombin required its proteolytic activity, and therefore the mechanism whereby thrombin could activate its G-protein-coupled receptor by a proteolytic mechanism was not initially self-evident. It was thus remarkable that receptor activation turned out to involve the proteolytic unmasking of an N-terminal receptor sequence that becomes a tethered ligand. The revealed N-terminal sequence then binds to the receptor extracellular domains to trigger receptor signalling (Vu et al. 1991) (Fig. 2.1, panel a). Because of this novel mechanism of activation, the receptor for thrombin was designated as a “proteinase-activated receptor” and was assigned the acronym “PAR” by the International Union of Pharmacology (Hollenberg and Compton 2002). The first thrombin-activated PAR to be cloned has now been designated as PAR₁.

A second remarkable observation described in the landmark manuscript reporting the cloning of human PAR₁ (Vu et al. 1991) was that a synthetic tetradecapeptide with a sequence matching that of the exposed tethered ligand (SFLLRNPNDKYKEPF) could also activate the receptor in the absence of proteolysis. It was soon realized that much shorter peptides, beginning with the sequence of human PAR₁, e.g. SFLLRN..., can function as surrogate activators of the receptor for thrombin in a variety of settings, without the need for receptor proteolysis (e.g. Fig. 2.1, panel b). These PAR-activating peptides (initially termed thrombin receptor-activating peptides or TRAPs; now termed PAR-APs), which mimic the ability of thrombin to activate PAR₁, soon revealed that in rat and rabbit platelets, the PAR₁-activating peptide (PAR₁-AP) did not cause a thrombin response (e.g. aggregation) (Kinlough-Rathbone et al. 1993). This result and other structure–activity studies with peptides based on the SFLLRN sequence provided evidence for the existence of thrombin receptor subtypes, not only in rodent platelets but also in rat vascular and gastric tissues (Hollenberg et al. 1993).

The continued work in the late 1990s to identify the non-PAR₁ receptor in rodent platelets responsible for the action of thrombin led ultimately to the discovery of PARs 3 and 4 (Ishihara et al. 1997; Kahn et al. 1998; Xu et al. 1998; Coughlin 2000, 2005). Concurrently, a homologous receptor activated by trypsin, but not thrombin, was fortuitously discovered in the course of screening a murine genomic library for the bovine substance K receptor (Nystedt et al. 1994). Each of these G-protein-coupled receptors (now designated PARs 1–4) has a unique N-terminal tethered ligand sequence unmasked by serine proteinase action, as summarized in Table 2.1 and depicted in Fig. 2.1. PARs 1, 3 and 4 have been found to be cleaved preferentially by thrombin, whereas PAR₂, not readily activated by thrombin, can be activated by trypsin, tryptase, kallikreins 5, 6 and 14 (Oikonomopoulou et al. 2006a), and by other serine proteinase members of the clotting cascade apart from thrombin (e.g., tissue factor-VIIa-Xa complex; activated protein C) (Coughlin 2000; Macfarlane et al. 2001; Hollenberg and Compton 2002; Ruf et al. 2003; Ossovskaya and Bunnett 2004; see chapter by Riewald in this volume). Although the signalling

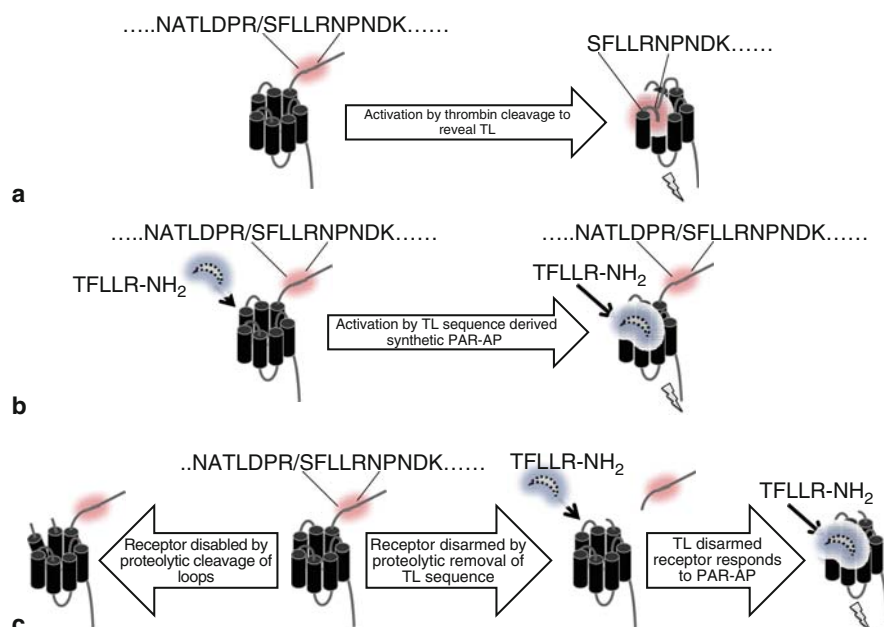


Fig. 2.1 Model for activation, disarming and disabling of human PAR₁. The scheme shows the proteolytic activation of human PAR₁ by the unmasking of its tethered ligand (TL) sequence (shaded domain). Once exposed by an activating proteinase (e.g. thrombin), the TL sequence binds to the receptor extracellular domain to trigger activation (*panel a, right-hand portion*). Alternatively (*panel b*), a synthetic peptide with a sequence representing the first five or six amino acids of the TL domain can activate the receptor without the need for proteolysis (e.g., the PAR-AP, TFLLR-NH₂; *arrow on right, panel b*). As shown in *panel c (right portion)*, a proteinase that cleaves downstream of the tethered ligand sequence can “disarm” the receptor, preventing activation by another enzyme; but the disarmed receptor can still respond to a PAR-activating peptide (*panel c, right portion*). However, if a proteinase cleaves other extracellular loops, the receptor becomes disabled and can no longer be activated by either a proteinase or a receptor-activating peptide (*panel c, left portion*)

properties of PAR₃ remain a bit of a puzzle, PARs 1, 2 and 4 have been found to signal via a G-protein-coupled mechanism involving the G_i or G_q and G_{12/13} family members. Currently, PAR₃ is thought to be a non-signalling “co-receptor” that facilitates the ability of thrombin to bind and activate PARs 1 and 4. Of importance for studying the potential physiological roles of PAR activation, it has proved possible to design synthetic peptides (PAR-APs) that can selectively activate each receptor, by mimicking the revealed tethered ligand sequences of PARs 1, 2 and 4. Appropriate standard inactive peptides have also been synthesized that cannot activate the PARs, serving as essential control reagents for the active peptides (Table 2.1). The PAR-activating peptides have paved the way in understanding the inflammatory and other effects that PAR activation can cause in vivo.

Table 2.1 Activation, disarming and blocking PARs by enzymes, PAR-activating peptides and antagonists: Relationship to revealed tethered ligand sequences

	PAR ₁	PAR ₂	PAR ₃	PAR ₄
Activating proteinases	Thrombin, trypsin, plasmin, HK14	Trypsin, tryptase, trypsin 2, matriptase, MT-serine protease 1, TF VIIa/Xa, PR3, HK5, HK6, HK14, Derp3, Derp9	Thrombin	Thrombin, trypsin, cathepsin-G
Human PAR cleavage site (/) and tethered ligand sequences (underlined)	LDPR/SFLLRN	SKGR/SLIGKV	PIK/TFRGAP	PAPR/GYPGQV
Disarming proteinases	Cathepsin-G, proteinase-3, elastase, plasmin, chymase	Tryptase, cathepsin-G, proteinase-3, elastase	Unknown	Unknown
Selective agonist peptides	TFLLLR-NH ₂	2-Furoyl-LIGRL-NH ₂ , SLIGRL-NH ₂ , SLIGKV-NH ₂	TL peptide not active. Activates PAR ₁ and PAR ₂	AYPGQV-NH ₂ , AYPGKF-NH ₂
Inactive control peptides	FTLLR-NH ₂	2-Furoyl-OLRGIL-NH ₂ , LSIGRL-NH ₂ , LSIGKV-NH ₂	None	YAPGQV-NH ₂ , YAPGKF-NH ₂
Antagonists	RWJ-56110 and RWJ-58259, SCH-205831, SCH5303048 SCH79797	ENMD-1068	None	<i>trans</i> -cinnamoyl-YPGKF-NH ₂ , P4pal-10

In addition to being activated by a variety of serine proteinases that can unmask the tethered ligand sequences (panel a, Fig. 2.1), PARs can be silenced or “disarmed” by proteinases that remove the tethered ligand sequence by cleaving at sites beyond the activating domain, as shown in panel c of Fig. 2.1. For example, trypsin, which can potentially activate both PARs 1 and 2, can prevent the ability of thrombin to activate PAR₁ presumably by removing the PAR₁ tethered ligand sequence (Kawabata et al. 1999). This disarming action of trypsin occurs at enzyme concentrations that are lower than those that can activate PAR₁ (see Fig. 3 in Kawabata et al. 1999). In a similar way, *Pseudomonas aeruginosa*, a complicating pathogen in the setting of cystic fibrosis, secretes an elastase that cleaves and removes the tethered ligand sequence from PAR₂, thereby disabling the receptor on lung epithelial cells (Dulon et al. 2005). The disabling of PAR₂ in this setting may contribute to the pathophysiology

of lung inflammation in this disease. Nonetheless, the disarmed receptor can still respond to the PAR-activating peptide (Fig. 2.1, panel c), as does the intact receptor (Fig. 2.1, panel b). In other instances, proteinase action may target not only the N-terminal tethered ligand domain but also the extracellular loops involved in signal generation. In such instances, the receptor would be silenced in a way that even the receptor-activating peptides cannot overcome (Fig. 2.1, panel c, left-hand portion). Thus, PARs can be said to have a variety of circulating agonists (i.e., serine proteinases that reveal the tethered ligand) as well as circulating functional antagonists that can truncate them downstream of their tethered ligands, thereby silencing the receptors. In certain settings *in vivo*, the ability of a proteinase to disarm a PAR may be as important physiologically as is the ability of an enzyme to activate a PAR.

2.3 Enzymes Other than Thrombin That Are Potential Physiological Regulators of PARs

As implied by the above section, the same PARs targeted by thrombin can also be regulated by either activation or disarming by other serine and non-serine proteinases. A question to ask is: Which of the proteinases that can be shown to regulate PARs *in vitro* might also function *in vivo*?

2.3.1 *Enzymes of the Coagulation Pathway*

Although thrombin is clearly accepted as a principal physiological regulator of PARs 1 and 4 (Coughlin 2000, 2005), particularly in terms of targeting the cardiovascular system (platelets, endothelial cells, vascular smooth muscle), it was not initially appreciated that other coagulation pathway enzymes such as factor Xa and activated protein C could target PAR₁, as well as PAR₂ (Ruf et al. 2003; Blanc-Brude et al. 2005; Versteeg and Ruf 2006; Bhattacharjee et al. 2008). Thus, in the counter-regulatory clotting cascade pathway, both plasmin and activated protein C can in principle affect the PARs (see chapter in this volume by Riewald). The action of plasmin on PARs is complicated, because this fibrinolytic enzyme can both disarm and activate PAR₁ (Kimura et al. 1996; Kuliopulos et al. 1999) and can activate PAR₄ (Quinton et al. 2004). Of note, plasmin can also regulate signalling via an apparently non-PAR mechanism in monocyte-macrophages by cleaving the annexin A2 heterotetramer (Laumonnier et al. 2006; Li et al. 2007). Activated protein C, via its endothelial adsorption site and a targeted interaction involving its exosite domain, can activate PAR₁ (Riewald et al. 2002; Yang et al. 2007; also, see chapter by Riewald in this volume). An interpretation of these data is complex, however, since the ability of annexin A2 to bind factor Xa facilitates the activation of PAR₁ (Bhattacharjee et al. 2008) and this type of role for annexin in the action of plasmin cannot be ruled out. Notwithstanding, in addition to thrombin, other enzymes of the

coagulation cascade known to be activated in a number of settings in vivo can be considered as physiological regulators of PAR activity.

2.3.2 Proteinases of the Gastrointestinal Tract

When discovered, PAR₂ was found to be particularly sensitive to activation by trypsin, whereas the receptor was not activated by thrombin (Nystedt et al. 1994). Because PAR₂ was subsequently localized at the apical membrane of rat enterocytes, it was proposed that trypsin present in the intestinal lumen may represent a physiological regulator of PAR₂ at this site (Kong et al. 1997). Since PARs 1 and 2 are known to be present in intestinal tissue, and since trypsin can both activate and inactivate PAR₁, depending on the trypsin concentration, intestinal serine proteinases can be important physiological regulators of several PARs, including those targeted by thrombin. Considerably elevated levels of serine proteinase activity have been observed in the colon tissue of a rat model of colitis, as well as in tissues from ulcerative colitis patients (Hawkins et al. 1997). Further, studies on intestinal biopsy samples from humans with inflammatory bowel syndrome have documented the release of serine proteinases that are able to activate PAR₂ (Cenac et al. 2007). The release of luminal serine proteinases that can activate PAR₂ has also been documented in a murine model of infectious colitis. In that study, not only were trypsin family members identified in the luminal contents by mass spectral analysis, but the administration of a trypsin inhibitor was able to mitigate the inflammatory process triggered by the infection (Hansen et al. 2005). Thus, it appears that endogenous trypsin-related colonic proteinases are able to activate PAR₂, known to be present on enterocytes. In principle, these same enzymes would be capable of mimicking thrombin's ability to activate either or both of PARs 1 or 4. Since a variety of other proteinases are also generated in the colon in inflammatory disease (e.g. cathepsins, metalloproteinases), it is highly likely that by either activating or disarming the PARs, these enzymes, in addition to thrombin, which would also very likely be present, will be found to play important inflammatory roles in colitis via the PARs (Vergnolle 2005).

2.3.3 PAR-Regulating Proteinases in the Central Nervous System

As discussed in greater detail in the chapter by Luo et al. in this volume, the central nervous system has been known for some time to produce not only its "own thrombin," but also a number of other serine proteinases with trypsin-like activity, such as mesotrypsin/trypsin IV, p22 and neurosin (kallikrein-related peptidase-6, or KLK6). Since both thrombin and its receptor can be up-regulated in the context of CNS pathology, like HIV encephalitis (Boven et al. 2003), it is possible that other serine proteinases may participate with thrombin to regulate CNS inflammation.

Given the PAR-regulating activities of both KLK6 (Oikonomopoulou et al. 2006a) and trypsin IV/mesotrypsin (Wang et al. 2006; Knecht et al. 2007), it is likely that these enzymes can play a role by targeting PARs in vivo. Again, the ability of the trypsin-related proteinases to activate or disarm the PARs in the CNS tissues will be an important issue. Our ability to detect active KLK6 (neurosin) in samples of human cerebrospinal fluid (Oikonomopoulou et al. 2008) adds support to the hypothesis that this PAR-regulating enzyme, like brain-localized trypsin IV, can play a physiological or pathophysiological role in inflammatory conditions of the central nervous system.

2.3.4 Immune-Cell-Derived Proteinases and PARs

Cells of the immune system both express functional PARs and produce proteinases that can potentially regulate PAR function. As summarized in more detail elsewhere, where the reader is referred to for a more extensive bibliography (Steinhoff et al. 2005; Ramachandran and Hollenberg 2008; Shpacovitch et al. 2008), PAR₁ expression has been found on many immune cells, including macrophages, monocytes, lymphocytes and mast cells. Other immune cells such as T cells and Jurkat-T leukemic cells are seen to be thrombin-responsive presumably via PAR₁. Since the data have been accumulated using cells and culture systems from a variety of species (humans, monkeys, mice, guinea pigs, rats), it is not yet clear that the expression and responsiveness of PAR₁ in all of these cells are the same across species. The expression of the other main target for thrombin, PAR₄, is less well characterized, except for its functional presence on human and rodent platelets. In particular, PAR₄ appears to play a key role in thrombin-regulated leukocyte rolling and adherence, as observed in rats by using an in vivo intravital microscopy system (Vergnolle et al. 2002). PAR₂ expression has been detected on mast cells, eosinophils, monocytes, macrophages and neutrophils, but the function of the PARs in these cells is not always clear.

The sources of proteinases that may regulate immune cell PARs in vivo are yet to be precisely determined. Clearly, thrombin is an accepted candidate to regulate PARs 1 and 4 on a variety of cells, since this enzyme can be activated in proximity to these inflammatory cells in vivo. However, the immune cells themselves are good candidates as physiological sources of PAR-regulating enzymes that can act in a paracrine or autocrine manner. For instance, T-cell-derived granzyme A can, like thrombin, activate PAR₁, whereas neutrophil elastase can disarm both PAR₁ and PAR₂. Cathepsin G, which like elastase also disarms PAR₁, can nonetheless mimic thrombin's action to trigger PAR₄, as can members of the trypsin family, in addition to pancreatic trypsin (trypsin I). Mast cells, known to be a source of tryptase and chymase, can also regulate PARs via the secretion of these enzymes. For instance, chymase activity can disarm PARs 1 and 2, whilst tryptase in certain settings can activate PAR₂. The overall impact of mast cell proteinases on PAR activity is difficult to determine, since there is considerable variation between species

(e.g. rodent vs. human) in the cellular content of proteinases. Notwithstanding, this brief discussion should be sufficient to indicate that in an inflammatory setting, with the influx of a complex mixture of immune cells, the local environment will no doubt contain a variety of enzymes in addition to thrombin that can regulate the activity of PARs.

2.3.5 Tumor-Derived Proteinases and a Possible Physiological Role for Kallikrein-Related Peptidases (KLKs) as PAR Regulators

Although matrix metalloproteinases have received much attention in the setting of cancer spread (López-Otín and Matrisian 2007), serine proteinases are also released in a tumour environment. Amongst the serine proteinases that have been linked to cancer-associated pathophysiology, the kallikrein-related peptidase (KLK) family of secreted enzymes, the best known of which is prostate-specific antigen (PSA) or kallikrein 3 (KLK3), has received significant attention (Borgono and Diamandis 2004; Borgono et al. 2004). Secreted as inactive zymogens, the human kallikreins can exhibit either trypsin- (12 family members) or chymotrypsin-like (3 family members) activity (Borgono et al. 2004). Several members of the family have been shown to possess clinical value as biomarkers for cancer diagnosis and prognosis (Borgono and Diamandis 2004). In this regard, PSA-KLK3 has been proved to be a valuable marker for prostate cancer diagnosis, together with another member of the family, KLK2 (Becker et al. 2001). Although the targets of the KLKs that regulate cell function are not clear, we hypothesized that similar to thrombin, the KLKs could signal by regulating PAR activity (Oikonomopoulou et al. 2006a). We found that all three of the kallikreins we evaluated (KLKs 5, 6 and 14) were able to increase intracellular Ca^{2+} in target cells and platelets by activating one or more of the PARs. Significantly, there were distinct differences between KLKs 5, 6 and 14 in terms of their selective actions on each of PARs 1, 2 and 4. For example, KLK14, like trypsin, could activate PARs 2 and 4; and depending on its concentration, could both activate (high concentrations) and disarm (low concentrations) PAR_1 , thereby preventing its activation by thrombin (Oikonomopoulou et al. 2006a). In contrast with KLK14, KLKs 5 and 6 preferentially activated PAR_2 , leaving PAR_4 intact. Thus, in vivo, like thrombin, but via a mechanism different from that of thrombin, KLK14 (but neither of KLKs 5 or 6) can be seen as a potential regulator of platelet function, exhibiting preferential activation of platelet PAR_4 . This action of KLK14, via PAR_4 , would release human platelet endostatin instead of VEGF, as thrombin would do by selectively activating PAR_1 (Ma et al. 2005). Furthermore, similar to trypsin, all of KLKs 5, 6 and 14 relaxed isolated rat aorta tissue, via the PAR_2 -mediated endothelium-dependent nitric oxide pathway (Oikonomopoulou et al. 2006a). Comparable results would be expected for human vascular tissue. Thus, all three KLKs (5, 6 and 14) can potentially have an impact on cardiovascular function that may be as important in certain settings as is the action of thrombin. Finally, as for trypsin, the intraplantar administration of KLK14 in vivo resulted in a significant

paw inflammatory response (Oikonomopoulou et al. 2006b). Further work will be required to evaluate the potential inflammatory and nociceptive roles that, similar to thrombin and trypsin, the kallikrein-related peptidases (KLKs) may play in vivo by regulating PARs.

Our data suggest that by activating PARs, tumour-related kallikreins, such as KLKs 5, 6 and 14, may play active roles in the setting of carcinogenesis. Because of the extensive tissue distribution of the KLKs, at sites where thrombin and trypsins may be absent, because the KLKs can participate in enzymatic activation reactions as complex as the clotting cascade involving thrombin, and because the KLKs can either activate or disarm/inactivate PARs, the kallikrein-related peptidase family (KLKs) are very likely physiological regulators of PAR signalling in vivo. The challenge that remains is to determine whether or not the high levels of KLKs detected by immunoassay in tumour-derived or body fluid samples represent enzymatically active species. Our recent data suggest that although the proportion of enzymatically active KLK is low, relative to the amount of inactive enzyme detected by immunoassay, the amounts of active enzyme produced by tumours could in theory be sufficient to regulate PAR activity (Oikonomopoulou et al. 2008).

No doubt, a variety of other proteinases produced in the setting of tumorigenesis will be able to regulate PAR activity in a way that mimics or blocks thrombin action. For instance, the metalloproteinase MMP1 has been found to be a tumour-cell-derived enzyme that can promote the invasive activity of cells because of the activation of PAR₁ (Boire et al. 2005). This effect of MMP1 that mimics thrombin action is particularly relevant in view of the invasive role that PAR₁ may play in the process of cancer cell growth and invasion (see chapter by Salah et al. in this volume). Another serine proteinase derived from mammary tumor cells, matriptase (now known as membrane-type (MT)-serine protease 1), can regulate PAR₂ activity (Takeuchi et al. 2000) and very likely, similar to trypsin, will be found to disarm PAR₁. In summary, tumor-derived proteinases, in addition to thrombin, very likely play important roles via the PARs in vivo.

2.3.6 Pathogen-Derived Proteinases and PAR Activation

Although most pathogens ultimately trigger a delayed immune response, it is becoming evident that in some situations, pathogen-derived proteinases can trigger an immediate innate immune response, in part by regulating PAR activity. For instance, the allergenic properties of the dust mite species *Dermatophagoides pteronyssinus* and *D. farinae* are now believed to involve not only protein antigens but also the action of the cysteine (Der p 1 and Der f 1) and serine (allergen groups 3, 6 and 9) proteinases. The allergen proteinases have been observed to disarm PAR₁ and to activate PAR₂ (Sun et al. 2001; Asokanathan et al. 2002; Adam et al. 2006). The proteinases in such allergens, like those from the common cockroach, *Blattella germanica*, participate in the allergenic response (Page et al. 2007), potentially by involving PAR activation (Page et al. 2003). What is evident is that activation of a

PAR, like PAR₂, can enhance the impact of asthmatic allergens (Ebeling et al. 2007). Periodontitis represents another inflammatory setting in which pathogen-derived proteinases can play a role (Lourbakos et al. 2001; Holzhausen et al. 2006). Importantly, although the arginine-specific cysteine proteinase from *Porphyromonas gingivalis* (an important pathogen of chronic periodontitis) was able to activate PAR₂ (Lourbakos et al. 2001; Holzhausen et al. 2006), an accompanying organism, *T. denticola*, that coexists with *P. gingivalis* in the inflammatory biofilm, produces a proteinase that can disable PARs (Holzhausen et al. 2006). Whether thrombin acting via PARs might also participate in a response to these organisms in vivo via PARs 1 and 4, or whether the proteinases such as elastase from invading pathogens, such as *Pseudomonas aeruginosa*, might block the actions of thrombin by disarming the PARs (Dulon et al. 2005) is an open question.

The pathogens themselves need not always be the source of the PAR-regulating proteinases. For instance, in a murine model of infectious colitis, the pathogen *Citrobacter rodentium* causes colon inflammation by a process that involves PAR₂ (Hansen et al. 2005). Although the initial hypothesis was that PAR₂ was being activated by a *Citrobacter*-derived proteinase, a molecular analysis of the enzymes induced in vivo in the colon by the infection revealed the up-regulation of murine proteinases (granzyme A and trypsin family members). Thus, host proteinases induced by pathogens, by regulating PARs, could in principle mimic processes that would otherwise be due to thrombin action.

The unifying theme of the information presented in the preceding sections is that in addition to the prototype PAR-activating enzymes, thrombin and trypsin, a variety of other proteinases can now be seen to play “hormonal” roles by regulating cell and tissue signals via the PARs. The examples provided so far are restricted to those sources from which the production of PAR-regulating proteinases has been verified in tissue or cell extracts. The challenge faced is to identify the specific proteinases, including thrombin, which may be responsible for modulating the inflammatory response via the PARs in vivo.

2.4 Receptor Dynamics and Cell Signalling: Enzyme-Mediated vs. Peptide-Mediated Activation

2.4.1 PAR-Mediated Signalling

As illustrated in Fig. 2.1, PARs can be activated either enzymatically or by the surrogate PAR-activating peptides. Indeed, it has been the receptor-selective PAR-activating peptides (Table 2.1) that have led the way in understanding the potential effects of activating PARs in vivo or in cell culture systems in vitro. Upon activation, the G-protein-coupled PARs can go on to generate cell signals via a number of different G-protein mediators (G_q , $G_{1/2}$, $G_{12/13}$), as illustrated in the chapter by Trejo in this volume and as described in greater detail elsewhere (Macfarlane et al. 2001;

Hollenberg and Compton 2002; Coughlin 2005; Steinhoff et al. 2005). The general turnover of PARs upon their activation is discussed in detail by Trejo (see chapter in this volume), with important differences noted in the dynamics of PARs 1 and 4 (Shapiro et al. 2000). Depending on the cell context in which the PARs are triggered, a wide variety of pleiotropic effects can ensue, including an inhibition of adenylyl cyclase ($G_i/G_{\beta\gamma}$), increases in intracellular calcium (G_q) along with the activation of protein kinase C, activation of non-receptor tyrosine kinases ($G_i/G_{\beta\gamma}$) and the regulation of Rho-mediated cytoskeletal events ($G_{12/13}$ -RhoGEFs). In particular, PAR₁, activated by thrombin, is able to trigger a number of “growth”-associated signal pathways, including the activation of the mitogen-activated protein kinase (MAPK) families (p42/44; p38, JNK) and trans-activation of growth factor receptors such as the one for EGF, by a mechanism that involves a metalloproteinase and/or the participation of Src-related kinases (Daub et al. 1996, 1997). Similar to the EGF receptor, the cMet receptor can also be trans-activated by thrombin action (Fischer et al. 2004). As for PAR₁, the activation of PARs 2 and 4 can also lead to increases in intracellular calcium (G_q), with an ensuing activation of protein kinase C and other calcium-stimulated enzymes, such as those regulated by calmodulin. Similarly, activation of PARs 2 and 4 can lead to the activation of “growth factor”-like pathways, involving the activation of MAPKs and transcriptional events. That said, the individual PARs can trigger both distinct and common signal pathways, depending on the cellular contexts in which signalling occurs. These distinct signal pathways presumably depend on the differences in the intracellular receptor sequences responsible for their G-protein interactions. This issue is complex, since evidence now indicates that the PARs can form heterodimers, for example, between PARs 1 and 4 (Leger et al. 2006) and between PARs 1 and 3 (McLaughlin et al. 2007). Since PARs 1 and 4 can signal via distinct pathways (Ma et al. 2005; Voss et al. 2007), whereas PAR₃ is unable to signal on its own, the implication of PAR dimer formation in terms of thrombin action remains to be explored in depth. Significantly, the dynamics of signalling, internalization and receptor degradation (discussed in detail in the chapter by Trejo) appear to differ for PARs 1 and 4, the main GPCR targets for thrombin (Shapiro et al. 2000). For PAR₂, a G-protein-independent, arrestin-dependent mechanism for signalling is also possible (DeFea 2008; Zoudilova et al. 2007). The arrestin-dependent mechanism can presumably mediate signalling via trypsin, which activates PAR₂; but it is not yet clear whether or not thrombin, acting via PARs 1 and 4, might use a comparable signal pathway.

2.4.2 PAR Activation by Enzyme vs. Peptide

As mentioned above and as will be discussed in the sections that follow, the PAR-activating peptides have been used extensively to assess the biological effects of PAR activation. These receptor agonists and their control peptides (Table 2.1) have been essential to distinguish the receptor-mediated processes from those non-PAR actions that the proteolytic enzyme activators can also have (see section on non-

PAR actions of thrombin later). However, since receptor activation by cleavage is an irreversible event (panel a, Fig. 2.1; and see chapter by Trejo in this volume), whereas the binding of the PAR-activating peptide is reversible, it might be expected that the result of activating the PARs with the PAR-APs might differ from activation by a proteinase. Data supporting this idea have come from work with thrombin and the PAR₁-activating peptides such as TFLLRNKPKD and SFLLRN (Vouret-Craviari et al. 1992, 1993; McLaughlin et al. 2005). These agonists appear to have differential effects on triggering elevations of intracellular calcium (G_q -mediated) vs. decreasing transendothelial electrical resistance, or triggering mitogenesis in cultured hamster fibroblasts, thereby implying a difference in signalling. We have suggested that this difference between signalling activated by thrombin and the PAR-activating peptide might be due to a cooperative action between the binding of the tethered ligand on the one hand and a non-catalytic domain of thrombin on the other. Indeed, when combined, a PAR-activating peptide and the thrombin-derived chemotactic peptide described by Bar-Shavit and colleagues (1984, 1986) can act synergistically to trigger hamster cell mitogenesis, whereas the thrombin-derived peptide on its own is inactive in terms of stimulating cell division (Hollenberg et al. 1996). One issue with these studies is that the peptide agonists used at that time might have been able to cross-activate PAR₂ as well as PAR₁ (Kawabata et al. 1999). Therefore, the interpretation of these data, that the peptide and thrombin signal differently (thrombin would not be able to activate PAR₂) must be accepted with caution. In previous work, it had been shown by site-directed mutagenesis of the receptor extracellular domains, that the site of receptor activation by a peptide agonist of PAR₁ differs from that of the tethered ligand revealed by thrombin action (Blackhart et al. 2000). Further confirmation of this concept has come from work with PAR₂, for which sequences that activate the receptor as trypsin-revealed tethered ligands (e.g. SLAAAA ...) cannot activate the receptor as synthetic peptides (e.g. SLAAAA-amide) (Al-Ani et al. 2004). In contrast with the results obtained using the PAR-APs, data obtained by activating cell or tissue systems with the enzymes, thrombin or trypsin, cannot be interpreted solely in terms of the activation of PARs. For that interpretation, work with PAR antagonists is essential to show that the enzyme actions are blocked. The PAR₁ antagonist that blocks the human receptor (SCH 79797) is readily available, but many early studies using thrombin as an agonist did not distinguish between the PAR-related and non-PAR actions of thrombin or trypsin. Currently, reagents are available to block PAR₄ (P4-pal10 pepducin: Covic et al. 2002; and trans-cinnamoyl-YPGKF-amide: Hollenberg and Saifeddine 2001) to evaluate a role for PAR₄ in thrombin-activated processes. Thus, the results obtained with the PAR-activating peptides can, with appropriate controls, be interpreted as representing the consequences of PAR activation.

The ability of thrombin and the PAR₁/PAR₂-activating peptides to trigger distinct responses in vascular tissues is illustrated by some of our preliminary data, shown in Fig. 2.2. In the intact porcine coronary preparation, thrombin causes an endothelium-dependent relaxation, as shown in tracing A of Fig. 2.2, but does not cause a contractile response. In contrast, the PAR₁-activating peptide, even at concentrations (1–5 μ M) well below its maximal contractile concentrations (10–20

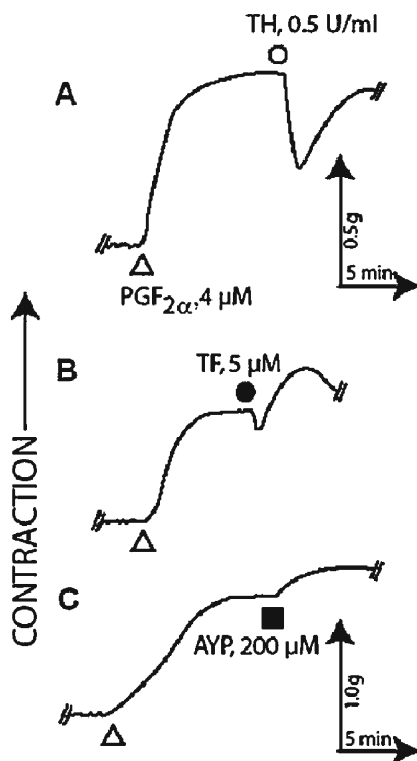


Fig. 2.2 Differential actions of thrombin and a PAR_1 -activating peptide in porcine coronary tissue. Porcine coronary artery rings with an intact endothelium were first precontracted with prostaglandin $\text{F}_{2\alpha}$ ($\text{PGF}_{2\alpha}$, 4 μM , all tracings) and were then exposed to the PAR -activating agonists: thrombin (TH, 0.5 U mL^{-1} or 5 nm , tracing A), the PAR_1 -selective agonist, TFLR- NH_2 (TF, 5 μM , tracing B), and the PAR_4 -selective agonist, AYPGKF- NH_2 (AYP, 200 μM), Tracing C). Tension (g) was monitored continuously. The scales for time (min) and tension (g) are shown by the insets; the double lines // signify washing of the tissues free of agonists

μM) causes principally a contractile response, preceded by a small relaxation (tracing B, Fig. 2.2). In contrast with the PAR_1 -activating peptide, a PAR_4 -activating peptide caused only a contractile response (Fig. 2.2, tracing C). Thus, in the porcine coronary preparation, the actions of thrombin were not mimicked by peptide activation of either of PARs 1 or 4. These observations using the receptor-selective PAR_1 agonist, TFLR-amide, reflect data obtained some time ago by Simonet and coworkers (1992). Those data need to be re-evaluated, since the peptide agonist used at the time might have activated both PAR_1 and PAR_2 in the vascular preparations studied (canine saphenous veins and coronary artery). These observations therefore merit further exploration. Our preliminary data, implying either a non- PAR mechanism for thrombin action in the coronary preparation, or pointing to an action of thrombin via PARs that cannot be mirrored by peptide activation, sound a cautionary note in interpreting

results obtained either with the PAR-APs or with thrombin. Thus, the results obtained with a proteinase agonist such as thrombin must be judiciously interpreted with the use of appropriate receptor antagonists, in terms of PAR-related and non-PAR responses. Given that an action of thrombin or other proteinase that is not mimicked by the PAR-APs may indeed involve PAR activation, it is to be expected that the proteinase agonists may stimulate PAR signalling pathways in addition to those activated by the peptides.

2.5 PAR Activation and the Inflammatory Actions of Thrombin

The discovery of thrombin in the late nineteenth century was due to its association with fibrin formation, implying a close link between its action and injury/inflammation. Considerable data accumulated over the century since thrombin's discovery, particularly within the past 20 years, have documented the ability of thrombin to trigger many of the responses associated with inflammation, including endothelial cell activation (P-selectin display, increased adhesion of leukocytes and platelets), along with increased vascular permeability (Malik and Fenton 1992), mast cell degranulation (Razin and Marx 1984), increased adhesion of neutrophils to the endothelium (Toothill et al. 1990), platelet aggregation and chemotaxis of neutrophils (Bizios et al. 1986) and the induction of cytokine release from epithelial and vascular smooth muscle (Kranzhofer et al. 1996) and endothelial cells (Stankova et al. 1995; Ueno et al. 1996). Subsequent to these observations, it was the availability of the receptor-selective PAR₁-activating peptides, along with PAR₁ null mice and PAR₁ antagonists, that established PAR₁ as the thrombin target potentially responsible for all the hallmarks of inflammation: increased blood flow and temperature, pain, oedema and loss of function. In sum, these inflammatory responses can be rationalized by the impact of PAR₁ activation on the endothelium and smooth muscle of blood vessels, nerve cells and bone cells, all of which have been found to possess functional PAR₁. Although less well documented, it is to be expected that the other PAR target of thrombin, PAR₄, will also play an ancillary role in the inflammatory response (Houle et al. 2005), in keeping with its independent action in human platelets to complement thrombin's concurrent activation of PAR₁. The rat paw oedema model of inflammation illustrates the way in which the actions of a PAR₁-activating peptide can be compared with the effects of thrombin *in vivo* (Vergnolle et al. 1999). Perhaps surprisingly, this inflammatory response to PAR₁ activation is to a large extent neurogenic in nature, depending on the ability of PAR₁-containing afferent spinal neurons to release substance P, which then activates the NK1 receptor on endothelial cells to promote oedema (de Garavilla et al. 2001). The action of thrombin in this oedema model is however complex. Thrombin, like the PAR₁-selective activating peptide, Ala-parafluoroPhe-Arg-Cha-Cit-Tyr-NH₂, causes inflammation, but when acting in combination is also able to diminish the inflammatory action of the peptide (Vergnolle et al.

1999). Although the anti-inflammatory target of thrombin is yet to be identified, our subsequent work demonstrates that it is not PAR₄, since a PAR₄-activating peptide also causes oedema in the rat paw model (Houle et al. 2005).

An important role for thrombin is well established in mediating neuro-inflammatory responses, and studies have revealed increased levels of thrombin at sites of injury (Smirnova et al. 1996) and in the proximity of amyloid plaques in the brains of patients with Alzheimer's disease (Akiyama et al. 1992). Recent studies have delineated the involvement of PAR₁ further in mediating many thrombin-mediated responses in the brain. Low concentrations of thrombin and the PAR₁ tethered ligand derived peptide SFLLRN were shown to reduce β -amyloid neurotoxicity and astrocyte stellation in an in vitro study using cultured neurons and astrocytes (Pike et al. 1996). The study, however, also found that in the presence of thrombin there was increased β -amyloid-stimulated secretion of basic fibroblast growth factor, an index of neurotoxicity (Pike et al. 1996), thus suggesting that thrombin, at least in part through PAR₁, can mediate both protective and degenerative responses in the setting of Alzheimer's disease. More recent studies focusing on the role of thrombin and PAR₁ in the setting of HIV-associated neurodegenerative disorders have shown that PAR₁ levels are elevated in the brains of HIV encephalitis patients (Boven et al. 2003). The study also confirmed that in cultured human astrocytes, thrombin and PAR₁-AP can stimulate increases in IL-1 β and nitric oxide synthase. In an in vitro setting, thrombin, through PAR₁, is also shown to activate microglial cells, stimulating calcium and MAPK signalling pathways dependent cell proliferation (Suo et al. 2002). As mentioned before, less is known about the role of the other thrombin receptor, PAR₄. However, it has been proposed that while PAR₁ is involved in mediating thrombin-stimulated microglial proliferation, PAR₄ mediates the ability of thrombin to stimulate the release of inflammatory mediators from microglial cells (Suo et al. 2003).

The involvement of PAR₄ in inflammatory responses was also revealed through observations in vivo that thrombin and PAR₄-AP, but not PAR₁-AP, can induce leukocyte rolling in rat mesenteric venules (Vergnolle et al. 2002). In an inflammatory setting, it has also been shown that PAR₄ expression can be induced in bronchial fibroblasts (Ramachandran et al. 2007) and on endothelial cells (Ritchie et al. 2007) which do not express the receptor under resting conditions, suggesting an involvement for this receptor in inflammatory processes.

2.6 Non-PAR Mechanisms of Cell Regulation Mediated by Thrombin and Other Proteinases

As mentioned in previous sections, a large number of studies documenting the actions of thrombin on cell and tissue targets were done before the PARs had been characterized and before reliable PAR₁/PAR₄ agonists or antagonists were readily available. Thus, in terms of thrombin action, not only is it impossible to determine which of its two PAR targets (PARs 1 or 4) may have been responsible for results documented in the literature; but it is also not at all certain that the thrombin effects

were due to PAR activation. The following sections deal with mechanisms other than PAR activation that can mediate the actions of thrombin.

2.6.1 Signalling Targets That Are Not “Classical” Receptors

In addition to the PARs, a variety of “non-receptor” targets can also result in signalling by proteinases. For instance, disruption of extracellular matrix-integrin signalling by cleaving either the integrins, or the matrix with which they interact, would in principle alter cell behaviour. In this regard, the ability of thrombin to activate endothelial-cell-derived metalloproteinases (Lafleur et al. 2001), which in turn may remodel the extracellular matrix, could in principle lead to PAR-independent signalling. A mechanism for proteinase-triggered signalling dealt with briefly in a previous section involves the action of plasmin, which in addition to regulating the PAR activity (Kuliopulos et al. 1999), can signal via a mechanism involving plasmin-mediated proteolysis of annexin A2 that in turn causes the activation of signal transduction pathways. The annexin-A2-mediated signal causes chemotaxis in human monocytes (Laumonier et al. 2006; Li et al. 2007). It can be presumed that serine proteinases other than plasmin will also be found to regulate cell behaviour via this novel annexin A2 proteolytic process. The signalling mechanism whereby annexin cleavage regulates chemotaxis or other cell responses remains to be determined. Whether thrombin at high concentrations might mimic the annexin A2 cleavage caused by plasmin, in the manner that plasmin mimics the action of thrombin on the PARs, is also an issue to consider.

2.6.2 Non-catalytic Mechanisms for Proteinase-Mediated Signalling

In addition to their catalytic actions, proteinases can also regulate processes via their non-catalytic domains, as discussed in detail for thrombin in the chapter by Olszewska-Pazdrak et al. in this volume. As outlined in that chapter, apart from its ability to signal by cleaving the PARs, thrombin can also yield, from within its sequence, chemotactic-mitogenic peptides released by proteolytic processing (Bar-Shavit et al. 1984, 1986). These thrombin-derived peptides can cause effects by interacting with cell surface receptors that are not PARs (Glenn et al. 1988). The presence of an Arg-Gly-Asp sequence in the peptide described by Glenn et al. (1988) implies a role for integrins in its action, since integrins can bind to sequences containing an RGD motif. The ability of proteinases to affect signalling via their non-catalytic domains is an issue that can often be overlooked.

To sum up, the above sections illustrate that, apart from targeting the PARs, proteinases can affect cell signalling by cleaving a diverse set of other substrates. This diversity of hormone-like signalling mechanisms triggered by proteinases is exceeded only by the diversity of the proteinase families themselves.

2.6.3 *Thrombin-Mediated Generation of Agonists from Fibrin and Other Substrates*

In addition to stimulating cells by cleaving signalling targets, as outlined in the above sections, proteinases can also generate peptide agonists by processing both pro-hormones (e.g., the production of interleukin-1 β by the action of interleukin-converting enzyme/caspase-1 on pro-IL1 β) and other substrates. For instance, in an inflammatory setting that results in fibrin deposition, this substrate can yield proteolytic cleavage products with permeability-enhancing, chemotactic and vasoactive properties (Sueishi et al. 1981; Saldeen et al. 1985; Senior et al. 1986). In particular, the action of thrombin or plasmin on fibrin(ogen) can yield a 14-amino-acid chemotactic peptide sequence, termed human fibrinopeptide B, from the N-terminus of the B β -chains (Kay et al. 1973, 1974; McKenzie et al. 1975). To date, the human fibrinopeptide B receptor responsible for its actions has not been identified, although a binding site with an affinity in the range of 7 μ M has been observed in responsive T cells (Esch and Thomas 1990). Thus, the effects of thrombin via the release of the fibrin(ogen)-derived peptides can in some ways mimic the effects of thrombin-mediated activation of PARs. No doubt, at concentrations generated in the circulation in certain settings, thrombin could in principle cleave other substrates to release biologically active peptides. In a similar way, other serine proteinases could trigger cellular responses in a restricted environment via the release of active peptides from fibrin(ogen) or other substrates that accumulate at sites of inflammation. These active peptides generated by thrombin and other proteinases at the sites of inflammation could regulate many cellular processes and merit scrutiny in future work.

2.7 Therapeutic Implications of Thrombin Action via PAR and Non-PAR Mechanisms

Primarily because of its involvement in cardiovascular pathophysiology, thrombin has for some time been an important therapeutic target. Since the inhibition of the catalytic activity of thrombin itself blocks all of its effects, except possibly those mediated by the thrombin-derived peptides, thrombin inhibitors have proved successful for anticoagulant therapy. Nonetheless, the inhibition of thrombin can promote a bleeding diathesis, and it has thus been of considerable interest to develop thrombin receptor antagonists that would not have that drawback. The search for thrombin receptor inhibitors, described by Chen et al. in this volume, began at a time when it was not appreciated that PAR₁ was not the only target. Now that receptor-selective inhibitors are available for PAR₁ (see chapter 12) and PAR₄ (Hollenberg and Saifeddine 2001; Covic et al. 2002; Leger et al. 2006), a key therapeutic question to ask is: Which is better, inhibiting thrombin or blocking its receptor (or both)? The answers to this question are linked not only to the molecular mechanisms of thrombin action but also to the clinical contexts in which blocking thrombin action is of therapeutic value.

2.7.1 Targeting Thrombin and Other Serine Proteinases

The therapeutic success of targeting proteinases is perhaps best illustrated by the development of the zinc metalloproteinase-selective angiotensin-converting enzyme (ACE) inhibitors (Cushman and Ondetti 1999). A most important issue in the design of ACE inhibitors was the development of enzyme selectivity and potency. As recounted by Cushman and Ondetti (1999), it was the biology of snake venom, with its content of ACE-inhibitory peptides that led the way to the design of non-peptide inhibitors, such as Captopril. Similarly for thrombin, it has been the naturally occurring inhibitors such as heparin and the leech-derived anticoagulant, hirudin, that have paved the way. Although the development of orally available thrombin inhibitors is beyond the scope of this chapter, it is sufficient to note that the use of crystallographic (Bode et al. 1989; Rydel et al. 1990) and other biochemical approaches (Kelly et al. 1992) has resulted in the synthesis of enzyme inhibitors that are quite selective for this serine proteinase over others, despite its general structural homology with enzymes such as chymotrypsin (Bode et al. 1989). This issue is dealt with in part in the chapter by Mousa in this volume. That said, the thrombin inhibitors target uncomplexed α -thrombin and may not efficiently inhibit either catalytically active thrombin species generated by enzymes other than factor Xa or thrombin complexed with thrombomodulin. Thus, even in the presence of clinically useful thrombin inhibitors, isoforms of thrombin (e.g., catalytically active species generated from prothrombin by enzymes other than factor Xa) could in principle generate active fibrin fragments, as outlined earlier, or could potentially still activate PARs 1 and 4, albeit with a lowered efficiency. These possibilities have not yet been explored in any depth. Other than thrombin, targeting other enzymes that are also capable of activating the PARs represents a substantial challenge, because of the diversity of the serine proteinases that can do so (ranging from trypsin family to the kallikrein-like peptidases, or KLKs) and the difficulty in designing high-potency orally available enzyme-selective inhibitors. For instance, enzyme inhibitors such as Nafamostat, aimed at blocking tryptase action, are also very efficient in inhibiting other serine proteinases and cannot be said to be enzyme-selective. Notwithstanding, the thrombin-targeted enzyme inhibitors can be considered as a suitable alternative to the vitamin K antagonists (e.g. warfarin) that have for some time been in general use as therapeutically successful anticoagulants, particularly for individuals at risk for venous thrombosis.

2.7.2 Targeting the PARs

In view of the potential clinical complications resulting from warfarin-related anticoagulants or from thrombin inhibitors, the development of PAR antagonists that would act downstream of enzyme-mediated activation appears attractive. As described in the chapter by Chen et al. in this volume, there has been considerable success in developing PAR₁ antagonists to a level of clinical utility by the

Johnson & Johnson team (RWJ-56110 and RWJ-58259) (Andrade-Gordon et al. 1999; Zhang et al. 2001; Maryanoff et al. 2003) and by the Schering group (Chackalamannil et al. 2005). The RWJ compounds, although effective in non-human models of thrombosis (Derian et al. 2003), may not be clinically suitable as general systemic antithrombotics, but may prove successful as local inhibitors of thrombosis, e.g., for arterial stent adducts. The orally active PAR₁ antagonist developed by Schering (PAR₁ antagonist 55 or SCH205831) inhibits PAR₁ by competitively inhibiting tethered ligand binding site (Chackalamannil et al. 2005). A related PAR₁ antagonist, SCH5303048, is currently the subject of a phase II clinical trial, providing hope for the availability of an effective orally available PAR₁-targeted anti-thrombotic compound (Camerer 2007; http://www.sch-plough.com/schering_plough/news/release.jsp?releaseID=977603). There has been a significant, albeit limited, success in developing PAR₄ antagonists (Hollenberg and Saifeddine 2001; Covic et al. 2002), but these agents are not orally available since they are peptides, and their potencies are not yet sufficiently high to be considered for clinical use. Nonetheless, these peptide-based PAR₄ antagonists have value in animal models of disease to evaluate the participation or not of PAR₄ function in vivo (Slofstra et al. 2007; Strande et al. 2008). Since human platelets can be activated by either of PARs 1 or 4, a combined use of dual receptor inhibitors might be necessary for clinical situations to limit platelet activation.

To sum up, in terms of the role that thrombin plays in inflammatory processes, arguments can be made for the use of both thrombin-targeted and PAR-targeted inhibitors in a number of therapeutic settings. The choice of agent will be dictated largely by the clinical setting for blocking thrombin's actions (e.g., in the setting of venous thrombosis vs. the occurrence of platelet dysfunction during bypass surgery). Further, the presence in an inflammatory milieu of a wide spectrum of proteinases in addition to thrombin that can regulate PAR activity, as discussed previously in this chapter, would argue in favour of using both thrombin and PAR inhibitors. As also pointed out in previous sections, the actions of thrombin can be both inflammatory (e.g., thrombin-triggered platelet PAR₁/PAR₄ activation in an acute haemorrhage setting), and anti-inflammatory or anabolic (e.g., activation of protein C and triggering of growth factor pathways). Thus, a question to ask is: Might there be a selected time frame, early in an inflammatory event in which blocking thrombin action (e.g. both enzyme and PARs) would be therapeutically beneficial, whereas at a later time point (e.g. resolving inflammation), thrombin and/or PAR_{1/4} inhibition would be counterproductive? Further, in certain situations, such as the development and invasion of tumors (see chapter in this volume by Salah et al.), it may prove essential to block thrombin and its target PARs continuously (see chapter by Petralia and Kakkar). This question deals with what might be considered to be the "fourth" dimension (i.e. timing) of therapeutic efficacy, a consideration that is not frequently discussed. This "timing" aspect of therapeutics may be of particular importance when dealing with the actions of thrombin.

2.8 Summary

In terms of a “hormone-like” agonist, thrombin can be seen as a prototype for proteinases that can signal to cells and tissues via a number of complementary mechanisms, acting either in an autocrine or in a paracrine manner in the immediate cell environment, or at a distance, via the circulation. The discovery of the PARs, prompted by the search for the thrombin receptor responsible for stimulating mitogenesis and platelet aggregation, has led to an entirely new paradigm not only for the action of thrombin but also for the actions that many other proteinases may play in inflammatory and other physiological processes. Further, the lessons from studying thrombin’s modes of action have illustrated several mechanisms other than PAR activation or silencing that also generate cell signals. These mechanisms include both targeting non-PAR membrane constituents that, like the PARs, can signal directly (e.g., integrin-mediated signalling), and the generation of peptide agonists derived from pro-hormone-like molecules such as pro-IL-1 β or from substrates in the inflammatory milieu, such as fibrin. These signalling mechanisms can be seen to play a role in a variety of inflammatory diseases ranging from asthma to arthritis. Thus, the proteinases themselves or their target receptors, such as the PARs, can be considered as attractive therapeutic targets. Notwithstanding, the complex and diverse mechanisms that proteinases use to generate cell signals require a thoughtful approach to developing new treatment modalities, in terms of which target to aim for (e.g. PAR or its activating proteinase) and which time frame might be appropriate for antagonism in a given pathophysiological process (the time factor, or the “fourth dimension” of therapeutics). It is hoped that the information discussed in this chapter, with reference to other contributions in this book, will provide a stimulus for developing new treatments for inflammatory diseases. One looks forward with great expectations to the novel therapeutic directions that will be developed from the lessons that thrombin has taughtus.

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Chapter 3

Regulation of Thrombin Receptor Signaling

JoAnn Trejo

Abstract Thrombin, a coagulant protease, is generated at sites of vascular injury and elicits cellular responses critical for haemostasis and thrombosis, as well as inflammation and proliferation. Protease-activated receptors (PARs) are G-protein-coupled receptors that account for the majority of thrombin's effects on cells. PAR₁, the prototype for this family, is the predominant mediator of thrombin signaling in most cell types. PAR₃ and PAR₄ also respond to thrombin and are differentially expressed in various cell types. PARs are uniquely activated by proteolysis, which results in the generation of a tethered ligand that cannot diffuse away, unlike normal reversibly activated G-protein-coupled receptors. Since PARs are irreversibly activated, signaling must be tightly regulated. Desensitization and trafficking of proteolytically activated PARs control the magnitude, duration and spatial aspects of receptor signaling. Thrombin also elicits cell-type-specific response through the activation of distinct PARs and G-protein subtypes. Thus, elucidating the mechanisms that regulate PAR signaling in various cell types is critical for understanding their biological function. Here, I discuss our current understanding of the regulation of thrombin receptor signaling.

3.1 Introduction

Thrombin, the main effector protease of the coagulation cascade, is generated in response to vascular injury and in thrombotic disease, and drives fibrin deposition and platelet activation, which are critical for haemostasis and thrombosis (Coughlin 2005; Morrissey 2004). Thrombin-elicited cellular responses have also been implicated in inflammation, blood vessel formation and cancer progression (Arora et al. 2007; Coughlin 2005). Thus, an understanding of thrombin signaling

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is critical for developing new strategies that can be used in the prevention and treatment of thrombin-related vascular diseases and cancer progression. This is of particular importance since there is no drug currently available that selectively blocks thrombin receptor signaling.

Protease-activated receptors (PARs) are a small family of G-protein-coupled receptors (GPCRs) that mediate most, if not all, thrombin responses in cells. PAR₁, the prototype for this family, is the predominant mediator of thrombin signaling in human platelets, endothelial cells, fibroblasts and smooth muscle cells. PAR₃ and PAR₄ also respond to thrombin and are differentially expressed in various cell types. The proteolytic nature of PAR activation, which results in irreversible activation, is distinct from most GPCRs. Thus, PAR signaling is tightly regulated. Desensitization and trafficking of proteolytically activated PARs control the magnitude, duration and spatial aspects of receptor signaling. Several recent studies have revealed novel endocytic sorting mechanisms that regulate PAR signaling. Here, I discuss our current understanding of the regulation of thrombin receptor signaling in various cell types.

3.2 Cell-Type-Specific Expression of Thrombin Receptors

There are four PAR members, encoded by distinct genes in the GPCR superfamily, which is the largest family of cell surface signaling receptors in the human genome. PAR₁, PAR₃ and PAR₄ are activated by thrombin, whereas PAR₂ is activated by trypsin-like serine proteases, but not by thrombin (Nystedt et al. 1994). PAR₁ was the first thrombin receptor discovered and accounts for the majority of effects elicited by thrombin in many cell types, including human platelets. Hence, PAR₁ was originally dubbed the thrombin receptor. The search for other thrombin receptors was prompted by studies on PAR₁-knockout mice. Coughlin and colleagues demonstrated that fibroblasts derived from PAR₁-knockout mice were unresponsive to thrombin, whereas PAR₁-null platelets responded normally to thrombin (Connolly et al. 1996; Trejo et al. 1996). These unexpected findings led to the search and identification of mouse PAR₃ and PAR₄ and the discovery of species-specific differences in thrombin receptor expression in platelets and other cell types.

Several studies have clearly established that PAR₁ and PAR₄ are the functional thrombin receptors in human platelets, whereas PAR₃ and PAR₄ mediate thrombin signaling in mouse platelets (Ishihara et al. 1997; Kahn et al. 1998; Xu et al. 1998). Thrombin receptors are also differentially expressed in other cell types. In mouse endothelial cells, PAR₁ and PAR₄ mediate thrombin signaling (Kataoka et al. 2003). By contrast, PAR₃ is expressed together with PAR₁, but not PAR₄, in human endothelial cells (O'Brien et al. 2000). PAR₁ is also expressed in fibroblasts, smooth muscle cells, sensory neurons and glial cells; however, the expression of PAR₃ and PAR₄ in these cell types is less clearly defined.

3.3 Thrombin Receptor Activation and Signaling

3.3.1 Proteolytic Mechanism of Thrombin Receptor Activation

The activation of PARs by thrombin occurs through an unusual irreversible proteolytic mechanism (Fig. 3.1). The activation mechanism of PAR₁ has been most extensively studied. Thrombin binds to and cleaves PAR₁ with exquisite specificity. The PAR₁ N-terminal LDPRS residues are essential for thrombin recognition and cleavage (Vu et al. 1991a,b). A second interaction between thrombin's anion-binding exosite and an acidic region C-terminal to the PAR₁ cleavage site termed the "hirudin-like"

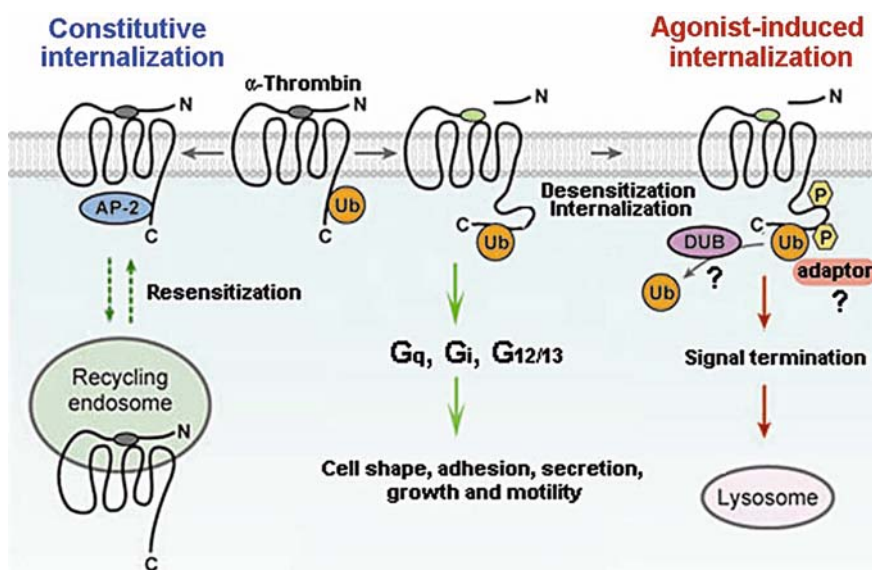


Fig. 3.1 Model of PAR₁ signaling and trafficking. PAR₁ displays constitutive and agonist-induced internalization. Constitutive internalization of PAR₁ requires AP-2 and is critical for cellular resensitization to thrombin signaling. Thrombin cleaves and irreversibly activates PAR₁ through a proteolytic mechanism. Once activated, PAR₁ couples to G_q, G_i, and G_{12/13} and promotes diverse cellular responses. PAR₁ signaling is controlled by multiple regulatory mechanisms, including desensitization and receptor trafficking. Phosphorylation and arrestins contribute to rapid desensitization of PAR₁ signaling. Internalization and lysosomal sorting are also critical for termination of PAR₁ signaling. Recent studies indicate that PAR₁ is basally ubiquitinated and that ubiquitination negatively regulates receptor constitutive internalization. Interestingly, ubiquitination is also important for specifying a distinct clathrin adaptor important for activated receptor internalization. The identity of the novel clathrin adaptor that regulates activated PAR₁ internalization remains to be determined. After activation, PAR₁ is de-ubiquitinated and sorted from endosomes to lysosomes and rapidly degraded. The identities of the ubiquitinating and de-ubiquitinating enzymes (DUB) and endocytic adaptor proteins that mediate ubiquitin-independent endocytic sorting of activated PAR₁ are not known

sequence increases its affinity for and remarkable potency towards PAR₁. Thrombin activates PAR₁ by cleaving an N-terminal peptide bond, which results in the formation of a new N-terminus that acts as a tethered ligand and binds intramolecularly to the receptor to trigger transmembrane signaling (Chen et al. 1994; Vu et al. 1991a). Synthetic peptides, which represent the newly formed amino terminus of the receptor, activate PAR₁ independent of protease and receptor cleavage.

Thrombin activation of PAR₃ may vary from this model. Human PAR₃ is proteolytically activated by thrombin when ectopically expressed in COS-7 cells (Ishihara et al. 1997). However, synthetic peptide agonists that mimic the putative PAR₃ tethered ligand unmasked by thrombin fail to activate the receptor. Thus, activation of PAR₃ may occur through alternative mechanisms. Interestingly, mouse PAR₃ does not appear to signal alone, but instead serves as a cofactor for cleavage and activation of PAR₄ (Nakanishi-Matsui et al. 2000). PAR₃ binds to and localizes thrombin for activation of PAR₄, a receptor that has low affinity for thrombin. The hirudin-like sequence, which confers high-affinity binding of thrombin to the receptor, is absent in the PAR₄ N-terminal domain. Thus, PAR₄ requires higher thrombin concentrations for activation, compared with other receptors (Kahn et al. 1999).

3.3.2 *Thrombin Receptor Signaling to Heterotrimeric G-Proteins*

PARs are likely to elicit signaling responses similar to the classic paradigm established for other GPCRs. That is, upon ligand activation of PARs, conformational changes in the receptor promote interaction with heterotrimeric G-proteins (Oldham and Hamm 2008). Heterotrimeric G-proteins are grouped into four main classes – $G\alpha_i$, $G\alpha_{i/o}$, $G\alpha_q$ and $G\alpha_{12/13}$, based on sequence similarity and regulation of specific signaling effectors. In the inactive state the $G\alpha$ -subunit is bound to GDP and $G\beta\gamma$ -subunits. Activated GPCRs promote exchange of GDP for GTP on the $G\alpha$ -subunit by inducing a conformational change in the G-protein and each activated $G\alpha$ -GTP and liberated $G\beta\gamma$ signal to downstream effectors. The dissociation of GDP from the $G\alpha$ -subunit is the rate-limiting step in G-protein activation. The deactivation of G-protein signaling is mediated by the intrinsic hydrolysis of GTP to GDP by the $G\alpha$ -subunit. The rate of GTP hydrolysis is considerably enhanced by regulators of G-protein signaling proteins, which stabilize the $G\alpha$ -GTP transition state intermediate and thereby function as GTPase-accelerating proteins. The inactive GDP-bound $G\alpha$ -subunit then reassociates with $G\beta\gamma$, and thereby terminates signaling.

Similar to other GPCRs, PAR₁ couples to multiple G-protein subtypes and promotes diverse cellular responses (Fig. 3.1). PAR₁ activation induces calcium mobilization, mitogen-activated protein (MAP) kinase signaling, Rho guanine nucleotide exchange factor (GEF)-mediated Rho and Rac signaling and regulation of many other effectors. Several early studies indicated that PAR₁ couples to inhibition of cAMP accumulation through $G\alpha_i$ and stimulates phospholipase C (PLC)-catalyzed hydrolysis of phosphoinositides through $G\alpha_q$ (Baffy et al. 1994; Benka et al. 1995;

Offermanns et al. 1997). Other studies have illustrated coupling of PAR₁ to $G\alpha_{12/13}$, which leads to activation of Rho GEFs, induction of cytoskeletal changes and PLC- ϵ activation (Klages et al. 1999; Lopez et al. 2001; Mao et al. 1998).

Human PAR₃ couples to $G\alpha_{16}$, a member of the $G\alpha_q$ class of G-proteins, in transfected COS-7 cells (Ishihara et al. 1997). However, whether endogenous PAR₃ signals similarly to other G-proteins is not known. Interestingly, mouse PAR₃ has not been shown to signal to G-proteins but rather binds and localizes thrombin for activation of PAR₄. PAR₄ couples to $G\alpha_q$ and $G\alpha_{12/13}$, but not to $G\alpha_i$, at least in fibroblasts (Faruqi et al. 2000). In many cases, the intracytosolic loops of GPCRs confer receptor-G-protein coupling; however, the specific residues that define the interface between PAR and contact with multiple G-protein subtypes are yet to be determined. Moreover, the extent to which PARs couple to distinct G-protein subtypes in a particular cell type is also influenced by the G-protein and effector repertoire expressed in the cells, but other regulatory mechanisms are likely to exist.

3.3.3 Cell-Type-Specific Thrombin Receptor Signaling

Thrombin elicits cell-type-specific responses through activation of distinct PARs and G-protein subtypes. The use of PAR knockout mice and blocking antibodies has been invaluable in delineating the roles of specific PARs in platelet activation and thrombosis. In human platelets, activation of PAR₁ with low thrombin concentrations is sufficient to trigger secretion and aggregation (Brass et al. 1992; Hung et al. 1992). However, PAR₄ mediates platelet activation at high thrombin concentrations in the absence of PAR₁ function (Kahn et al. 1999). The contribution of PAR₄ to normal platelet activation by thrombin generated physiologically is not known. In contrast to human platelets, PAR₄ is necessary for thrombin activation of mouse platelets (Kahn et al. 1998), which express PAR₃ and PAR₄ and not PAR₁. In this system, PAR₃ does not appear to mediate signaling, but instead facilitates thrombin activation of PAR₄. Indeed, platelets derived from PAR₄-deficient mice are unresponsive to thrombin even at supraphysiological concentrations (Sambrano et al. 2001).

Thrombin activation of platelets induces platelet shape change, release of platelet granule content containing adenosine diphosphate (ADP) and thromboxane A₂, and integrin-mediated aggregation. ADP and thromboxane A₂ further act on platelets, creating a positive feedback loop to amplify platelet activation. In mouse platelets, $G\alpha_q$ signaling is essential for thrombin-stimulated platelet secretion and aggregation, but is dispensable for platelet shape change induced by thrombin (Offermanns et al. 1997). Rather, low thrombin concentrations activate $G\alpha_{13}$ signaling to promote platelet aggregation and shape change; however, $G\alpha_{13}$ is not essential for these responses at high thrombin concentrations (Moers et al. 2003). In contrast to $G\alpha_{13}$, $G\alpha_{12}$ is not critical for thrombin signaling in platelets. Interestingly, thrombin signaling via $G\alpha_{z/\beta}$ synergizes with other platelet agonists to promote platelet activation.

In endothelial cells, thrombin activation triggers a host of cellular responses that contribute to haemostasis, inflammation and vascular development. Activation of PAR₁ on endothelial cells induces expression of adhesion molecules, disruption of

endothelial cell barrier, as well as cell migration and release of growth factors and cytokines. Thrombin signaling to $G\alpha_{12/13}$ and Rho-GEF-mediated activation of Rho are critical for endothelial cell barrier permeability (Gilchrist et al. 2001; Wojciak-Stothard and Ridley 2002). Increases in intracellular calcium induced by thrombin activation of PAR_1 promoted $G\alpha_q$ signaling also contribute to endothelial cell permeability in some, but not all, endothelial cell types, whereas $G\alpha_i$ does not appear to positively regulate permeability in most endothelial cell types (McLaughlin et al. 2005; Tiruppathi et al. 2002). PAR_1 function in endothelial cells is also important for the formation and maintenance of blood vessels during embryonic development. About half of *Par1*-null mice die at midgestation owing to abnormal vascular development, whereas reexpression of PAR_1 in endothelial cells prevents death (Connolly et al. 1996; Griffin et al. 2001). Recent studies also indicate that $G\alpha_{13}$ is a critical mediator of PAR_1 signaling in endothelial cells during development (Ruppel et al. 2005). Mouse embryos derived from endothelial-cell-specific $G\alpha_{13}$ knockouts displayed a phenotype that resembles PAR_1 deficiency, and restoration of $G\alpha_{13}$ expression rescued this phenotype.

Thrombin activation of PARs on fibroblasts and smooth muscle cells induces growth factor and matrix production, migration and proliferation, which may contribute to both normal wound healing and pathological proliferative responses such as restenosis and atherosclerosis. Trejo et al. 1996 previously demonstrated that PAR_1 is both necessary and sufficient for thrombin activation of extracellular-signal regulated kinases 1 and 2 (ERK1 and -2), important members of the MAP kinase family, and mitogenesis in fibroblasts. Moreover, PAR_1 signaling to a $G\alpha_q$ -like G-protein, protein kinase C, and c-Raf and to a $G\alpha_i$ -like G-protein-mediated activation of MAP kinase in fibroblasts expressing the endogenous receptor. These findings suggest that multiple signaling pathways are important for PAR_1 -mediated cellular proliferation. The majority of thrombin effects on smooth muscle cells appear to involve signaling by PAR_1 (McNamara et al. 1992), which has limited expression in normal vascular smooth muscle cells but is increased in cells localized to vascular lesions.

Thrombin activation of PAR_1 promotes mast cell degranulation (Cirino et al. 1996) and T-lymphocyte activation and induction of cytokine production (Mari et al. 1994). The activation of PAR_1 by thrombin in sensory neurons is associated with neurogenic inflammation, edema and hyperalgesia (Traynelis and Trejo 2007). In glial cells, thrombin activation of PAR_1 stimulates proliferation and release of neuroactive agents. The roles of PAR_3 and PAR_4 in cell types other than platelets remain obscure.

3.4 Regulation of Thrombin Receptor Signaling

3.4.1 Thrombin Receptor Desensitization

Thrombin irreversibly activates PARs, thus the mechanisms that contribute to the termination of signaling are critical determinants of the magnitude and duration of thrombin responses in cells. Desensitization, internalization and down-regulation,

three temporally distinct processes, mediate termination of signaling by most reversibly activated GPCRs. In the classic paradigm, GPCRs are initially desensitized by rapid phosphorylation of activated receptors by G-protein-coupled receptor kinases (GRKs) and other kinases (Krupnick and Benovic 1998). Phosphorylation enhances receptor affinity for arrestin, and arrestin binding prevents receptor–G-protein interaction, thereby uncoupling the receptor from signaling. Arrestin also interacts with components of the endocytic machinery to facilitate GPCR internalization, and thereby removes activated receptor from signaling effectors at the plasma membrane. Within endosomes, receptor dissociates from their ligands, becomes dephosphorylated, and then returns to the cell surface in a state capable of responding to ligand.

Phosphorylation of activated PAR₁ appears to be important for rapid termination of signaling. GRK3 and GRK5 enhance activated PAR₁ phosphorylation and markedly inhibit signaling (Ishii et al. 1994; Tiruppathi et al. 2000). A mutant PAR₁ in which all serines and threonines within the cytoplasmic tail were converted to alanines is defective in phosphorylation and insensitive to GRK3-promoted inhibition of phosphoinositide hydrolysis (Ishii et al. 1994; Shapiro et al. 1996). However, this mutant still conferred concentration-dependent responses to thrombin, suggesting that other termination mechanisms must exist. Paing et al. (2002) found that arrestins also contribute to PAR₁ desensitization. The β -arrestin isoforms 1 and 2 (also known as arrestin 2 and 3) are ubiquitously expressed and bind to most activated and phosphorylated GPCRs to mediate desensitization and internalization. Interestingly, signaling by PAR₁ appears to be more effectively regulated by β -arrestin 1 than by β -arrestin 2 through a process that does not require receptor phosphorylation (Chen et al. 2004; Paing et al. 2002). Thus, PAR₁ signaling is controlled by multiple regulatory mechanisms that include both cytoplasmic tail phosphorylation and phosphorylation-independent binding of β -arrestin 1. The molecular basis for the differential regulation of PAR₁ signaling by the individual arrestin isoforms is not known. In addition, PAR₁ couples to $G\alpha_q$, $G\alpha_i$, and $G\alpha_{12/13}$, and whether arrestins and phosphorylation are the only components that regulate PAR₁ coupling to these distinct G-protein subtypes remains to be determined. The roles of phosphorylation and arrestins in regulation of PAR₃ and PAR₄ signaling remain to be defined.

In human platelets, activated PAR₁ signaling is rapidly desensitized (Molino et al. 1997). However, activated PAR₁ displays minimal internalization in human platelets. Thus, the majority of cleaved PAR₁ is retained on the platelet surface, suggesting that desensitization is sufficient to shut off PAR₁ signaling. In contrast to PAR₁, activated PAR₄ signaling is sustained in platelets and other cell types, possibly because of a lack of receptor phosphorylation and/or a slower rate of internalization (Covic et al. 2000; Shapiro et al. 2000). Unlike platelets, which presumably respond to thrombin only once, endothelial cells, fibroblasts and other cell types are exposed to thrombin repeatedly and need to recover thrombin signaling in a timely manner. This appears to be accomplished by movement of uncleaved PAR₁ from an internal pool to the cell surface, which permits rapid recovery of thrombin signaling independent of de novo receptor synthesis

(Hein et al. 1994; Paing et al. 2006). The mechanisms responsible for PAR₁ internalization and recycling are discussed below.

3.4.2 *Thrombin Receptor Internalization*

Owing to the irreversible nature of PAR activation, trafficking events are also critical for regulation of thrombin signaling. PAR₁ displays two modes of trafficking important for the fidelity of thrombin signaling (Fig. 3.1). Unactivated PAR₁ cycles constitutively between the cell surface and an intracellular compartment, generating a protected pool that replenishes the cell surface after protease exposure and leads to rapid resensitization independently of de novo receptor synthesis (Hein et al. 1994; Paing et al. 2006). Activated PAR₁, by contrast, is internalized, sorted predominantly to lysosomes and degraded (Hoxie et al. 1993; Trejo and Coughlin 1999). Internalization of irreversibly activated PAR₁ removes it from signaling effectors at the plasma membrane, and sorting of PAR₁ from endosomes to lysosomes prevents it from returning to the cell surface and continuing to signal, terminating signaling (Trejo et al. 1998).

Clathrin-mediated endocytosis is responsible for internalization of most GPCRs (Marchese et al. 2008). Clathrin-coated pits form at plasma membrane sites enriched in phosphatidylinositol (4,5)-bisphosphate. Clathrin, adaptor proteins and dozens of regulatory proteins coordinate the assembly and invagination of clathrin-coated pits through a highly regulated and dynamic process (Edeling et al. 2006). The GTPase dynamin is essential for release of clathrin-coated pits from the plasma membrane (Schmid 1997). Clathrin-coated pits are high-capacity carriers that efficiently internalize many different types of cargo. Clathrin adaptors recognize short linear peptide sequences as well as phosphorylated and ubiquitinated cargo. The function of clathrin adaptors is to enrich select cargo within a forming clathrin-coated pit.

Paing and coworkers previously demonstrated that unlike most reversibly activated GPCRs, activated PAR₁ is internalized through a clathrin- and dynamin-dependent pathway that is independent of arrestins (Paing et al. 2002). In mouse embryo fibroblasts derived from β -arrestin 1 and 2 double knockout mice, activated PAR₁ was rapidly recruited to clathrin-coated pits and internalized normally. However, internalization of PAR₁ was blocked by dominant-negative dynamin and agents that disrupt clathrin assembly (Paing et al. 2002). These findings strongly suggest that arrestins are not essential for PAR₁ internalization; however, activated receptor internalization still required phosphorylation. Arrestin-independent PAR₁ internalization has also been observed in other cell types (Chen et al. 2004; Paing et al. 2006). Thus, clathrin adaptors other than arrestin are likely to function as critical mediators of PAR₁ internalization.

The adaptor protein complex-2 (AP-2) is a plasma-membrane-localized clathrin adaptor composed of α , β_2 , μ_2 and σ_2 adaptin subunits that recognizes tyrosine- and dileucine-based motifs. The μ_2 -subunit of AP-2 binds directly to tyrosine-based

Y-X-X-Ø motifs localized within the cytosolic regions of integral membrane proteins to facilitate internalization. The activity of this type of motif requires that the critical tyrosine remain unphosphorylated. A bioinformatics analysis of the PAR₁ cytoplasmic tail sequences revealed the identity of two highly conserved tyrosine-based motifs. Paing et al. (2006) showed that the μ_2 -subunit of AP-2 binds directly to the distal tyrosine-based motif within the cytoplasmic tail of PAR₁ and is essential for constitutive internalization and for recovery of thrombin signaling in endothelial cells and other cell types (Fig. 3.1). Interestingly, internalization of activated PAR₁ through clathrin-coated pits remained intact in AP-2-deficient cells, suggesting that constitutive and activated receptor internalization are regulated by different endocytic machineries.

In recent studies Wolfe et al. (2007) discovered that PAR₁ is basally ubiquitinated and that ubiquitination regulates receptor internalization (Fig. 3.1). Interestingly, the major sites of PAR₁ ubiquitination occur at highly conserved lysine residues localized within the cytoplasmic tail tyrosine-based motif, the critical binding site of the μ_2 -subunit of AP-2 (Paing et al. 2006). These findings suggest that ubiquitination of PAR₁ may preclude AP-2 binding. Moreover, in the absence of ubiquitination, PAR₁ constitutive internalization is significantly enhanced, whereas fusion of ubiquitin to the cytoplasmic tail of PAR₁ reduced constitutive internalization (Wolfe et al. 2007). Thus, ubiquitination may provide a mechanism for retaining unactivated PAR₁ on the cell surface. Ubiquitination of PAR₁ is also important for specifying a distinct clathrin adaptor for activated receptor internalization that occurs independent of arrestins and AP-2. Studies using genetic deletions and RNAi silencing of genes have demonstrated a critical role for the clathrin adaptor proteins epsin and eps15 in mediating internalization of ubiquitinated cargo through clathrin-coated pits. The role of epsin and eps15 in the novel regulation of PAR₁ internalization by ubiquitination remains to be determined.

Interestingly, activated PAR₄ displays a slow rate of internalization and fails to undergo phosphorylation after activation, which is thought to contribute to sustained signaling by this receptor (Shapiro et al. 2000). Moreover, the mechanisms responsible for PAR₄ desensitization and internalization are yet to be determined. In addition, the mechanisms that regulate cleaved PAR₃ are not known.

3.4.3 *Thrombin Receptor Down-Regulation*

In contrast to most GPCRs, irreversibly activated PAR₁ is internalized, sorted predominantly to lysosomes and degraded (Hoxie et al. 1993; Trejo and Coughlin 1999). Internalization and lysosomal sorting of PAR₁ are critical to keep irreversibly proteolytically activated receptor from remaining at or returning to the cell surface and continuing to signal (Trejo and Coughlin 1999; Trejo et al. 1998). Previous studies unveiled a novel function for sorting nexins in lysosomal trafficking of proteolytically activated PAR₁. Agonist-induced PAR₁ lysosomal degradation is

impaired in sorting nexin 1 (SNX1) deficient cells or in cells in which endogenous SNX1 localization is disrupted (Gullapalli et al. 2006; Wang et al. 2002). SNX1 binds to the tubular portion of early endosomes and facilitates the “pinching-off” of endosomal vesicles through a process that involves other as-yet-unidentified endocytic adaptor proteins (Carlton et al. 2004). More recent work indicates that activated PAR₁ traffics from endosomes to lysosomes independent of ubiquitination and Hrs and Tsg101, components of the ubiquitin-dependent endosomal-sorting complex required for transport (ESCRT) machinery (Gullapalli et al. 2006; Wolfe et al. 2007). Indeed, after activation PAR₁ is deubiquitinated (Wolfe et al. 2007), suggesting that deubiquitinated, rather than ubiquitinated, PAR₁ transits through the endosomal–lysosomal system (Fig. 3.1). The identities of the endocytic adaptor proteins and ubiquitin-modifying machinery that mediate ubiquitin-independent lysosomal sorting of PAR₁ remain to be determined. The pathways responsible for PAR₃ and PAR₄ down-regulation have not been investigated.

3.5 PAR Activation and Signaling by Other Proteases

In addition to thrombin, other proteases can cleave and activate PARs. The upstream coagulant protease factor Xa cleaves and activates PAR₁ as a monomer or in a complex with tissue factor, a single spanning membrane protein, and FVIIa (Camerer et al. 2000; Riewald and Ruf 2001). Factor Xa can also cleave and activate PAR₄. The anti-coagulant protease activated protein C (APC) also elicits cellular responses through the activation of PAR₁. In human cultured endothelial cells, APC appears to act through PAR₁, but whether PAR₁ is the only mediator of APC responses *in vivo* is a subject of some controversy (Mosnier et al. 2007). Protein C, the precursor of APC, is localized to the endothelial cell surface by binding to the endothelial protein C receptor (EPCR) and is then cleaved and activated by thrombin bound to thrombomodulin. Although APC and thrombin are both thought to proteolytically activate PAR₁ on endothelial cells, they promote anti-inflammatory and proinflammatory responses, respectively (Feistritzer and Riewald 2005). Intriguingly, concentrations of low thrombin and high APC can reverse these opposing cellular responses, suggesting that the level of receptor activation is important for conferring specific cellular responses. In addition, it is not known whether regulation of PAR₁ signaling following activation by thrombin vs. APC is similar.

The mechanistic basis for how activation of the same receptor by two different proteases elicits distinct cellular responses remains to be determined. The compartmentalization of PARs and distinct G-protein subtypes in lipid rafts such as caveolae might confer PAR₁-G-protein selectivity and explain why APC bound to endothelial protein C receptor is markedly less efficient at stimulating Gα_q-mediated responses but promotes ERK1,2 signaling comparable to that elicited by thrombin (Feistritzer and Riewald 2005; Ludeman et al. 2005). Alternatively, specific proteases may stabilize distinct conformational states of PAR₁ that selectively couple to distinct G-protein subtypes. Indeed, PAR₁ appears to display differential G-protein coupling

when activated proteolytically by its tethered ligand vs. “free” synthetic peptide agonists (McLaughlin et al. 2005). Clearly, a more thorough understanding of the molecular mechanisms that dictate PAR₁-G-protein selectivity is needed.

Plasmin can also cleave PAR₁, which activates or incapacitates the receptor, depending on the position of the cleavage site (Kuliopulos et al. 1999). Recently, the matrix metalloproteinase-1 (MMP-1) (also known as interstitial collagenase) was reported to cleave and activate PAR₁ (Boire et al. 2005). However, the mechanism by which MMP-1 acts on PAR₁ to generate a functional ligand and/or signaling response remains to be determined (Nesi and Fragai 2007). Moreover, some proteases, including certain MMPs, disable PARs by cleaving downstream of the activation site, resulting in loss of the tethered ligand domain (Ludeman et al. 2004), and thus revealing a potentially important mechanism for regulation of PAR signaling in various cell types.

3.6 Conclusions

Thrombin receptors are uniquely activated by proteolytic cleavage, which results in irreversible activation, unlike normal ligand-activated GPCRs. Thus, rapid desensitization and receptor trafficking tightly regulate PAR signaling. The temporal and spatial regulation of PAR signaling is crucial for a variety of biological responses, including proper growth and cell migration. Recent studies have advanced our understanding of the molecular mechanisms that control PAR₁ constitutive endocytosis and its importance in the control of cellular resensitization. The mechanisms that control activated PAR₁ internalization remain unclear. The mechanisms that control signaling and trafficking of other thrombin receptors are yet to be fully elucidated and are also vital to our understanding of thrombin signaling. Finally, the molecular mechanisms that regulate protease and cell-type-specific signaling mediated by PARs remain largely undefined and are critical for understanding PAR biological function in various tissues.

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Chapter 4

Thrombin and Activated Protein C: Integrated to Regulate Vascular Physiology

Matthias Riewald

Abstract Thrombin has long been known to be a dual-faced molecule. On one hand, thrombin is the key procoagulant effector of the blood coagulation system; on the other, it is the only known physiological activator of the anticoagulant protein C pathway. This chapter will summarize recent results that thrombin and the protein C system are highly integrated to regulate not only thrombotic responses but also other aspects of vascular physiology that likely play key roles in the regulation of inflammatory responses.

4.1 The Protein C Pathway Is Localized to the Endothelial Cell Surface and Limits Thrombin Generation Through Negative Feedback

The serine protease activated protein C (APC) is an important physiological regulator of blood coagulation because it controls conversion of prothrombin to thrombin. APC is evolutionarily closely related to thrombin with a similar domain structure. Zymogen protein C (PC) has an N-terminal domain containing γ -carboxylated glutamic acid (Gla), two epidermal growth factor (EGF)-like domains, and a serine protease domain. The calcium binding Gla domain is formed by vitamin-K-dependent posttranslational carboxylation of glutamic acid residues, and this domain mediates binding of PC/APC to negatively charged phospholipid membranes and to the endothelial cell protein C receptor (EPCR). EPCR is a transmembrane receptor with a very short cytoplasmic domain and an extracellular domain that recruits and positions both PC and APC on the cell surface and activation of EPCR-bound PC is enhanced (Fig. 4.1) (Regan et al. 1997; Esmon et al. 1999).

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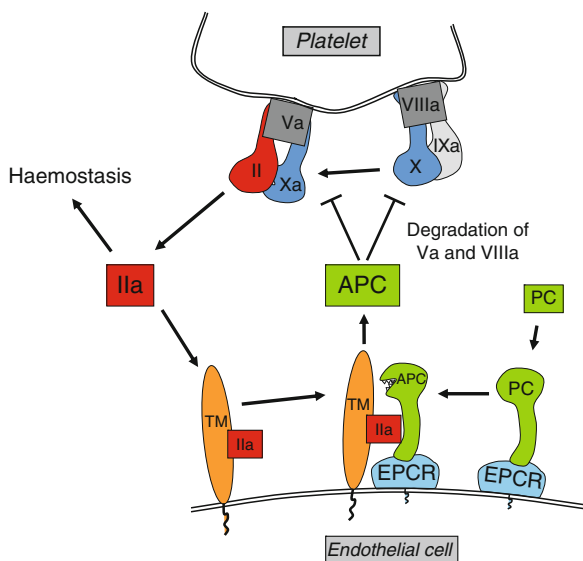


Fig. 4.1 APC downregulates thrombin formation in a negative feedback loop. Protein C (PC) is activated on the endothelial cell surface by thrombin (IIa) bound to thrombomodulin (TM). Binding of PC to its endothelial cell receptor (EPCR) enhances activation by thrombin–TM. The generated activated PC (APC) proteolytically inactivates the activated cofactors Va and VIIIa in the thrombin generation pathway

Thrombomodulin, another transmembrane receptor on the endothelial cell surface, is involved in PC activation. Thrombomodulin consists of an extracellular N-terminal lectin-like domain, followed by six EGF-like domains, a transmembrane domain, and a cytoplasmic C-terminal tail. Binding of thrombin to EGF domains 5 and 6 through its exosite 1 shifts thrombin's specificity from procoagulant functions towards activation of the anticoagulant PC. Proteolytic cleavage of EPCR-bound PC by thrombomodulin-bound thrombin results in the release of the activation peptide and the serine protease domain of PC is converted into its active conformation. The generated APC dissociates from EPCR and, together with its cofactor protein S, irreversibly inactivates the activated forms of factors V and VIII, thus inhibiting the prothrombinase and tenase complexes and limiting further thrombin generation in a negative feedback loop (Esmon 1995, 2003; Dahlback and Villoutreix 2005; Mosnier and Griffin 2006) (Fig. 4.1).

Both thrombomodulin and EPCR are expressed on vascular endothelial cells and localize the PC activation pathway to the endothelial cell surface. Thrombomodulin expression is especially high in the microvasculature whereas EPCR is highly expressed in larger vessels (Laszik et al. 1997). The anticoagulant negative feedback provided by the PC pathway is clearly of critical importance because patients with PC deficiency or with a variant of factor Va that cannot be efficiently inactivated by APC (factor V Leiden) have an elevated risk of thrombosis (Dahlback 1995).

4.2 APC Has Protective Effects in Systemic Inflammation That Are Independent of Its Anticoagulant Function

The proteases and receptor proteins involved in the PC pathway are all closely related to molecules of the innate immune system. Thrombin and APC are closely related to mannan-binding lectin serine proteases of the complement system; thrombomodulin is homologous to the complement C1q receptor; and EPCR is a member of the MHC class I/CD1 family. It is thus not surprising that numerous two-way interactions between coagulation and inflammation have been discovered (Opal 2000; Levi et al. 2004; Esmon 2005). Inflammation not only leads to activation of blood coagulation but products of the coagulation system in turn affect inflammatory responses. Bacterial septicemia has provided the most convincing example for an association of coagulation pathways with inflammation *in vivo*. In a lethal baboon sepsis model, inhibitors of the upstream coagulation initiation complex show marked improvements in lethality (Taylor et al. 1991a, 1998; Creasey et al. 1993), whereas blocking thrombin generation efficiently blocked microthrombosis and consumptive coagulopathy but did not prevent lethality (Taylor et al. 1991b). These findings strongly indicate that the initiation phase of coagulation can sustain an inflammatory response independent of the downstream effector protease thrombin.

In contrast, studies in the baboon sepsis model demonstrated that the infusion of APC reduces organ failure and lethality independent of its anticoagulant action but dependent on EPCR binding (Taylor et al. 1987, 2000). Studies in rat and mouse models support the conclusion that APC protects against pulmonary vascular injury and hypotension in endotoxemia models through mechanisms that are independent of its anticoagulant function (Murakami et al. 1996, 1997; Isobe et al. 2001). Similar anticoagulation-independent protective effects of APC were also found in a model of renal ischemia–reperfusion injury (Mizutani et al. 2000). Genetically engineered mice with very low levels of PC have a predisposition not only for thrombosis but also for enhanced inflammatory responses (Ganopoulosky and Castellino 2004; Lay et al. 2005, 2007). Most important, infusion of APC has been shown to reduce mortality in patients with severe sepsis in a large multicenter study whereas other anticoagulants such as antithrombin III and tissue factor pathway inhibitor were ineffective (Bernard et al. 2001; Warren et al. 2001; Abraham et al. 2003). Recombinant APC is now the first compound that has been specifically approved to treat adult patients with severe sepsis. Not surprisingly, the question how these protective effects of APC in systemic inflammation are mediated has received a considerable amount of attention in recent years (Mosnier et al. 2007).

4.3 The Thrombin Receptor PAR₁ Mediates APC Signaling in Tissue Culture

To adapt to their extracellular environment, living cells have sensors for extracellular proteolytic activity, members of the protease-activated receptor (PAR) family (Coughlin 2000, 2005; P.J. O'Brien et al. 2001; Ossosvskaya and Bunnett 2004).

PARs are seven transmembrane domain, G-protein-coupled receptors that are activated by trypsin-like serine proteases. Proteolytic cleavage of a specific arginyl peptide bond in the amino-terminal exodomain leads to the exposure of a neoamino terminus that folds back and activates the receptor ("tethered ligand"). Proteases of the coagulation system are major activators of PARs, suggesting that PARs have evolved to regulate cellular functions associated with the hemostatic response to vascular injury. Of the four known human PARs, PAR₁, PAR₃, and PAR₄ are activated by thrombin, whereas PAR₂ can be activated by several proteases, including factor Xa, but not by thrombin.

Activation of PAR₁ by thrombin is highly efficient because thrombin is directly recruited and positioned for receptor cleavage through an interaction between its exosite 1 and a hirudin-like sequence in PAR₁'s N-terminal exodomain (Vu et al. 1991b). An extremely low thrombin concentration of only ~50 pM is sufficient to obtain half maximal responses through PAR₁ activation (Vu et al. 1991a). PAR₁ is expressed on most vascular cell types, including human platelets, and this receptor clearly evolved as a highly sensitive cellular sensor for thrombin activity.

A fibroblast-derived cell line from PAR₁-deficient mice expresses no members of the PAR family, and these cells were used in transfection studies to analyze whether PARs mediate cellular responses to APC. Expression of human PAR₁ and PAR₂ in the PAR-deficient cells demonstrated that both receptors can be proteolytically activated by APC if EPCR is coexpressed (Riewald et al. 2002). EPCR facilitates PAR cleavage by lower APC concentrations, by recruiting and positioning the protease to specific domains on the plasma membrane (Bae et al. 2007c). Human umbilical vein endothelial cells (HUVECs) express PAR₁, PAR₂, and EPCR. APC induced downstream signaling responses such as phosphorylation of mitogen-activated protein (MAP) kinases in HUVECs dependent on binding to EPCR. Unexpectedly, cleavage-blocking antibodies to PAR₁ prevented APC-dependent downstream signaling, indicating that responses to APC require PAR₁ cleavage whereas the endogenous endothelial cell PAR₂ cannot mediate APC signaling. Large-scale gene expression profiling of HUVECs in response to specific PAR₁ and PAR₂ agonist peptides and APC demonstrated that all the APC-induced transcripts were also induced by the PAR₁ agonist, and PAR₁ activation accounted for all analyzed effects of APC on gene expression, including the upregulation of several protective and antiapoptotic genes (Riewald et al. 2002). EPCR binding and PAR₁ cleavage are also required for the induction of calcium flux by APC in brain endothelial cells (Domotor et al. 2003). The surprising conclusion from these studies was that the prototypical thrombin receptor PAR₁ mediates APC signaling in endothelial cells. Thus, the anticoagulant APC uses the same receptor as does the procoagulant thrombin. Given that thrombin-PAR₁ signaling is known to lead to a proinflammatory phenotype of endothelial cells, it was difficult to explain how PAR₁ can possibly mediate anti-inflammatory effects of APC.

4.4 APC and Thrombin Can Mediate Opposite Cellular Responses in Endothelial Cells Through PAR₁ Activation

4.4.1 *Barrier Integrity*

Endothelial cells at the interface between circulating blood and tissues play a key role in inflammation, not only because they affect leukocyte trafficking in and out of the tissues but also because they form a barrier for soluble factors. Breakdown of this barrier plays a key role in inflammatory disorders such as sepsis. Thrombin-PAR₁ signaling causes a rapid and transient contraction and rounding of endothelial cells in a confluent monolayer that results in gap formation between cells and loss of barrier integrity (Laposata et al. 1983). Barrier disruptive signaling downstream from thrombin-activated PAR₁ is mediated by the G-protein α -subunits $G\alpha_{12/13}$ which regulate Rho activation and stress fiber formation (Vouret-Craviari et al. 1998). In contrast, incubation of an endothelial cell monolayer with APC leads to an increased barrier function and lower permeability for macromolecules (Zeng et al. 2004; Feistritzer and Riewald 2005; Finigan et al. 2005). The barrier-disruptive effect of thrombin is rapid and reversible, whereas the barrier-protective APC effect appears more slowly over several hours and is longer lasting. Importantly, barrier-protective APC signaling requires binding to EPCR and cleavage of PAR₁ (Fig. 4.2).

4.4.2 *Adhesion Molecule Expression*

Leukocyte transendothelial migration is a highly regulated process that involves rolling and tight adhesion on the endothelial cell surface and migration through the cell barrier along chemotactic gradients. The current concept is that leukocytes first interact with adhesion molecules E- and P-selectin and then with intracellular adhesion molecule 1 and vascular cell adhesion molecule 1 on the apical endothelial plasma membrane. They subsequently crawl to cell–cell junctions where they transmigrate between cells or they follow a transcellular route. Proinflammatory cytokines such as tumor necrosis factor alpha (TNF- α) strongly induce the expression of adhesion receptors on the endothelial cell surface, contributing to the recruitment of leukocytes from the blood into the extravascular space during inflammation. Results from animal models indicate that infusion of APC can inhibit adhesion of leukocytes to the endothelial cell surface (Hoffmann et al. 2004; Iba et al. 2005). In tissue culture, incubation of endothelial cells with APC strongly diminishes the TNF- α -mediated induction of several adhesion molecules, including E-selectin, intracellular adhesion molecule 1, and vascular cell adhesion molecule 1 (Joyce et al. 2001). Thus, APC can prevent a key proinflammatory phenotypic change in endothelial cells. This effect again requires both APC binding to EPCR

Bcl-2 family and inhibitor of apoptosis protein 1 (Joyce et al. 2001; Riewald et al. 2002). Potentially proapoptotic gene products that are downregulated by APC include the tumor suppressor p53, thrombospondin-1, and the TNF-related protein TRAIL (Joyce et al. 2001; Cheng et al. 2003; Riewald and Ruf 2005; L.A. O'Brien et al. 2007). These direct cytoprotective actions of APC-PAR₁ signaling likely contribute to neuroprotective effects of APC in animal models of stroke (Cheng et al. 2003; Guo et al. 2004). Thrombin-PAR₁ signaling is known to induce apoptosis in neuronal cells, epithelial cells, and cancer cell lines, but thrombin can also mediate antiapoptotic effects (Flynn and Buret 2004). Interestingly, even though thrombin and APC induced a similar set of genes in quiescent endothelial cells, the potentially proapoptotic thrombospondin-1 is upregulated by thrombin and downregulated by APC in TNF- α -perturbed endothelial cells in a PAR₁-dependent manner (Riewald and Ruf 2005; McLaughlin et al. 2005a).

4.5 Role of the Sphingosine-1-Phosphate Pathway in Mediating Protective Signaling by PAR₁

Sphingosine-1-phosphate (S1P) is a biologically active lipid that is generated by cellular sphingosine kinases. S1P signaling is mediated by the S1P receptor family of seven transmembrane G-protein-coupled receptors. S1P can induce responses in endothelial cells that resemble APC-mediated responses, including enhanced barrier function, downregulation of adhesion molecules, and antiapoptotic effects (Lee et al. 1999; McVerry and Garcia 2005; Whetzel et al. 2006). Indeed, EPCR-dependent protective effects of APC on the barrier integrity of an endothelial cell monolayer require sphingosine kinase 1 activity and expression of the S1P receptor 1 (S1P₁) (Feistritzer and Riewald 2005; Finigan et al. 2005). Sphingosine kinase 1 and S1P₁ are also required for the downregulation of TRAIL expression by APC (L.A. O'Brien et al. 2007). It is possible that S1P pathway signaling could play a role in other responses to APC, including protective effects on adhesion molecule expression, apoptosis, and most importantly in beneficial effects of APC in systemic inflammation. In models of endotoxin-induced acute lung injury infusion of S1P has indeed been shown to be protective (McVerry and Garcia 2005). Additional studies will be required to test whether protective *in vivo* effects of APC require cross-activation of this pathway, including the activation of endothelial cell S1P₁. It is important to keep in mind that S1P receptor agonists and APC signaling will likely target different cell populations *in vivo* even if S1P pathway cross-activation is indeed a general requirement for protective responses to APC in tissue culture. This is because *in vivo* the PC pathway depends on the expression of cellular cofactors such as EPCR and thrombomodulin. The relative specificity of PC pathway signaling for endothelial cells may avoid detrimental side effects of S1P receptor activation in other cell types in the treatment of inflammatory conditions.

Clearly, very little is known about the mechanism of S1P receptor cross-activation by APC. How exactly do sphingosine kinases and S1P₁ contribute to the signaling?

It is difficult to explain how the S1P pathway can be relevant for APC signaling because plasma contains large amounts of S1P. Plasma S1P is expected to be largely bound to plasma proteins, and one possibility is that autocrine S1P₁-dependent signaling of endothelial-cell-produced and locally secreted S1P is more efficient when compared to exogenous S1P. APC can induce colocalization of EPCR with S1P₁, and S1P₁ may also be activated through mechanisms that do not involve S1P binding, e.g., cross-phosphorylation events (Finigan et al. 2005).

4.6 Protective PAR₁ Signaling by APC Is Mechanistically Coupled to PC Activation by Thrombin

Given that EPCR binding supports both PC activation and APC signaling, an intriguing possibility is that autocrine APC signaling is directly coupled to the process of PC zymogen activation by the thrombin–thrombomodulin complex. Using a thrombin variant with a relative specificity that favors PC activation relative to PAR₁ activation, it has been established that the endogenous PC activation pathway on the endothelial cell surface is indeed linked to PAR₁-cleavage-dependent autocrine barrier protective signaling by the generated APC (Feistritzer et al. 2006). Consistent with these results, the receptors critical for APC generation and signaling, i.e., thrombomodulin, EPCR, and PAR₁, are colocalized in lipid rafts in the endothelial cell membrane (Bae et al. 2007c). The dissociation constant for the interaction of both PC and APC with EPCR is about 30 nM (Fukudome and Esmon 1994; Liaw et al. 2001). At the PC plasma concentration of ~80 nM (Griffin et al. 1981; Heeb et al. 1988), therefore, the majority of the endothelial cell surface EPCR is expected to be PC bound. This EPCR-bound PC is activated by thrombomodulin–thrombin and the generated APC can activate PAR₁ while still bound to EPCR. Thus, at least part of the endogenously generated APC can be directly channeled into the PAR₁-dependent signaling pathway.

Could it be that the PC pathway and not thrombin may emerge as the relevant activator of endothelial cell PAR₁ in response to low thrombin concentrations? It is possible that in vivo low concentrations of thrombin preferentially lead to PC activation and PAR₁-dependent protective signaling by the endogenously generated APC. Thrombin binding to thrombomodulin blocks thrombin's PAR₁-interactive exosite I, and blood flow could affect the relative efficiency of thrombin for thrombomodulin binding vs. PAR₁ cleavage. In addition, thrombin inhibitors and substrates, foremost fibrinogen, that occupy exosite I of thrombin are present in plasma at a high concentration and may shift thrombin responses from PAR₁ towards PC pathway activation. The concept that thrombin may be able to initiate protective PC pathway signaling without activating PARs is also supported by in vivo evidence. Infusion of a low concentration of thrombin leads to activation of the PC pathway and blocks the lethal inflammatory response to endotoxin in animal models without eliciting platelet activation, a highly sensitive PAR-dependent response (Taylor et al. 1984; Hanson et al. 1993).

The finding that PC activation on the endothelial cell surface leads to efficient protective APC signaling raises the possibility that the infusion of an “anticoagulant” thrombin variant may induce more powerful protective effects in patients with sepsis than does exogenous APC. Data from a baboon model indicate that infusion of such a PC activator thrombin analog W215A/E217A (WE) can be antithrombotic without detectable prothrombotic activity or bleeding complications (Gruber et al. 2002), suggesting that WE can be safely administered in vivo. Both thrombomodulin and EPCR expression on vascular endothelial cells are downregulated upon severe inflammation (Faust et al. 2001), and protective signaling in response to WE would be expected to depend on an intact capacity to activate PC on endothelial cells. PC level and the ability to generate APC vary widely and independently in adult patients with severe sepsis (Liaw et al. 2004). Based upon the plasma PC level and the capacity for PC activation in patients with sepsis, therapy could be tailored to different subgroups. Patients with a relatively normal capacity to activate PC might benefit from WE infusion to trigger powerful barrier protective effects through the endogenous PC activation pathway. Zymogen PC could be substituted in the subgroup of patients with low PC levels. On the other hand, patients with more severe disease and lower PC activation capacity may be more likely to respond to exogenous APC. Clinical studies suggest that only patients with severe sepsis and a high but not low risk of death respond favorably to therapy with exogenous APC (Bernard et al. 2001; Abraham et al. 2005). At least part of the reason for this finding may be that patients with a high risk of death are also expected to have on average lower levels of circulating PC and less competition for EPCR binding of the infused APC. A higher dose of APC may be required for protective effects in patients with relatively high PC levels, and infusion of a variant APC with reduced anticoagulant but normal anti-inflammatory activity (Mosnier et al. 2004) may help prevent hemorrhagic complications in these patients.

Thus, the finding that powerful autocrine barrier protective signaling is mechanistically linked to the endogenous PC activation pathway has important implications for novel approaches to treat patients with systemic inflammation.

4.7 Not PAR₁- or EPCR-Dependent Mechanisms for Signaling by the PC Pathway?

So far the only established signaling pathway for APC in endothelial cells involves EPCR-dependent cleavage of PAR₁. However, some data suggest that other receptors may mediate responses to APC, and it is likely that other signaling mechanisms will be discovered in the future. In one report PAR₁ blockade suppressed early responses to APC in HUVECs but did not prevent late MAP kinase activation and cell proliferation (Uchiba et al. 2004). Considering the less efficient blocking with a single anti-PAR₁ antibody and the very high APC concentration (300 nM) used in this study, physiological significance of these findings needs to be established.

Another study suggests that PAR₁-dependent APC signaling does not always require EPCR binding (L.A. O'Brien et al. 2007). The authors report that the down-regulation of TRAIL by APC required cleavage of PAR₁ but was independent of APC binding to EPCR. Surprisingly, the induction of mitogen-activated kinase phosphorylation and activation of the transcription factor EGR-1 by APC-PAR₁ signaling were also EPCR-independent whereas previous studies reported the same pathways to be dependent on EPCR (Riewald et al. 2002; Uchiba et al. 2004). These divergent results may be caused by experimental conditions and the use of different cell lines. EPCR colocalizes with PAR₁ in lipid rafts, and ligand binding to EPCR may modulate its compartmentalization and affect downstream signaling responses (Esmon et al. 1999; Finigan et al. 2005; Bae et al. 2007a,b). A novel cofactor for APC recruitment may lead to distinct signaling responses, and taken together, these results highlight that novel receptors and signaling pathways may be involved in protective APC signaling in endothelial cells.

4.8 Thrombin-PAR₁ and APC-PAR₁ Signaling in In Vivo Models of Inflammation

The prototypical thrombin receptor PAR₁ has originally been described as mediating proinflammatory signals (Coughlin and Camerer 2003). Consistent with this concept, thrombin-PAR₁ signaling is proinflammatory in mouse models of glomerulonephritis (Cunningham et al. 2000) and renal ischemia–reperfusion injury (Sevastos et al. 2007). In addition, PAR₁ has clearly proinflammatory effects in inflammatory bowel disease (Vergnolle et al. 2004). However, complete PAR₁ deficiency did not affect survival in mouse models of endotoxemia (Pawlinski et al. 2004; Camerer et al. 2006). This argues that in systemic inflammation any beneficial effects in the absence of proinflammatory PAR₁ signaling may be offset by the absence of cytoprotective PAR₁-dependent signaling. Recent results in mouse models indeed support the conclusion that PAR₁ has dual roles in systemic inflammation that may depend on the stage of the inflammatory response. Mortality reduction by injected APC in lipopolysaccharide-induced endotoxemia requires PAR₁ (Kerschen et al. 2007). The finding that a nonanticoagulant APC variant with normal cytoprotective activity is as effective as wild-type APC indicates that this effect is not indirectly mediated by downregulation of detrimental thrombin-PAR₁ signaling. In another study using a cecal ligation and puncture sepsis model a pepducin PAR₁ antagonist was highly beneficial to the survival of the animals when the antagonist was administered immediately after surgery but not when given at later time points (Kaneider et al. 2007). On the contrary, a pepducin PAR₁ agonist was beneficial when given at later time points, arguing that during the progression of the septic response in mice PAR₁ mediates detrimental effects in early stages but beneficial effects at later stages. Thus the concept that similar to thrombin itself the thrombin receptor PAR₁ is a dual-faced molecule that can mediate both proinflammatory and protective effects helps to clarify the complex roles of PAR₁ in vivo (Fig. 4.2).

4.9 How Can Activation of the Thrombin Receptor PAR₁ by the PC Pathway be of Physiological Relevance?

Details of the molecular mechanisms how PAR₁-activation-dependent signaling can lead to opposite biological effects are the subject of ongoing research in several laboratories, and many questions remain to be answered. An important issue is that it is difficult to envision how PAR₁ signaling by endogenously generated APC can be relevant given that thrombin, the only known physiological activator of PC, is a much more efficient PAR₁ activator than APC. In this section recent research addressing these questions will be briefly summarized.

4.9.1 PAR₁ Cleavage by APC Is Very Inefficient Compared with Thrombin

Thrombin is an extremely efficient PAR₁ activator, and studies analyzing cleavage of synthetic peptides corresponding to part of the PAR₁ N-terminal exodomain in solution phase demonstrated that APC cleaves at the same position as does thrombin (Parry et al. 1996; Kuliopulos et al. 1999), albeit with about 25,000 times lower catalytic efficiency (Parry et al. 1996; Mosnier et al. 2004). Efficient cleavage of substrate proteins in a physiological system depends on the proper orientation and presentation of enzyme and substrate. Thrombin is directly recruited and positioned for cleavage of PAR₁'s scissile bond by binding to the hirudin-like sequence in PAR₁'s N-terminal exodomain. In contrast, cleavage of the PAR₁ scissile bond by APC depends on binding to a separate cofactor, i.e. EPCR. EPCR-dependent cleavage of an overexpressed epitope-tagged PAR₁ construct by APC was still ~10⁴-fold less potent, compared to thrombin (Ludeman et al. 2005). The overexpressed construct may be cleaved preferentially by the cofactor-independent thrombin, and cleavage of endogenous PAR₁ by EPCR-bound APC is indeed more efficient (Bae et al. 2007c; Schuepbach et al. 2008). However, activation of PAR₁ on the endothelial cell surface by APC-EPCR is still 100–1,000-fold less efficient than by thrombin, dependent on the specific readout and the expression level of EPCR.

4.9.2 What Are Physiologically Relevant Concentrations of Thrombin and APC?

Thrombin activity in vivo is rigorously controlled to prevent excessive platelet activation/fibrin formation and the half life of thrombin in the circulation is estimated to be only a few seconds. However, it is not known how high the relevant thrombin concentration in the endothelial cell microenvironment may be. Data from mouse models argue that thrombin likely operates just above threshold levels for PAR

activation in vivo. In mouse platelets PAR_4 cleavage is required for thrombin signaling and PAR_3 acts as a nonsignaling cofactor that recruits thrombin and decreases the concentration required for half maximal signaling between 6- and 15-fold (Nakanishi-Matsui et al. 2000). However, PAR_3 - and PAR_4 -deficient mice showed similar degrees of bleeding time prolongation and protection in thrombosis models, indicating that an about tenfold decrease in platelet responsiveness to thrombin has the same effect on hemostasis and thrombosis as complete unresponsiveness (Sambrano et al. 2001; Weiss et al. 2002). Compared with thrombin, inhibition of APC by plasma protease inhibitors is much slower and there is evidence that the serpin antithrombin III specifically evolved to prevent inhibition of APC (Hopkins et al. 2000). Consequently, the half life of APC in the systemic circulation is relatively long (~10–30 min). Plasma levels of APC in patients with sepsis vary widely and have been estimated to be ~0.1–0.2 nM. It is likely that local concentrations in the microenvironment of the endothelial cell surface where APC is generated are significantly higher, especially under conditions of laminar flow. Taken together, this argues that APC concentrations relative to thrombin may be high enough in vivo to allow significant PC-pathway-mediated PAR_1 cleavage and signaling.

4.9.3 Are Dual Roles of PAR_1 Dependent on Kinetics of Receptor Activation?

Thrombin can induce opposite cellular effects dependent on the thrombin concentration. Exposure of hippocampal slice cultures to 50 pM thrombin significantly improved neuronal survival after oxygen and glucose deprivation whereas concentrations of 500 pM or higher did not affect neuronal survival or further impaired it (Strigrow et al. 2000). The intracellular Ca^{2+} signals induced by 50 and 500 pM thrombin were distinct, suggesting that different kinetics of PAR_1 activation can indeed lead to qualitatively different intracellular signals. Similarly, very low concentrations of thrombin in the range of 20–40 pM, but not higher concentrations, can mediate S1P-pathway-dependent barrier protective effects in endothelial cells (Feistritzer and Riewald 2005). Unfortunately, the majority of studies analyzing cellular effects of thrombin signaling used only relatively high thrombin concentrations. It will be interesting to establish whether low-dose thrombin can mimic protective effects of APC signaling, e.g., on adhesion receptor expression and apoptosis in endothelial cells.

4.9.4 What Is the Role of Membrane Compartmentalization of PAR_1 ?

Recent studies suggest that binding of the Gla-domain of PC or APC to EPCR may affect the localization of PAR_1 in the cell membrane and downstream responses after PAR_1 cleavage by either thrombin or APC. Ligand binding to EPCR recruits PAR_1 into a protective signaling pathway by enhancing coupling of PAR_1 to $\text{G}\alpha_i$

activation (Bae et al. 2007b,c). According to this model, different PAR₁ activators, including thrombin and APC, are expected to mediate protective effects as long as EPCR is bound by PC/APC. Given that the PC concentration in plasma is high enough to result in more than 50% receptor occupancy of endothelial cell EPCR, thrombin is expected to mediate mainly protective in vivo effects in endothelial cells through activation of G α_i -dependent protective pathways.

4.9.5 Surface Retention of APC-, but Not Thrombin-, Cleaved PAR₁

APC can mediate barrier protective signaling and detectable PAR₁ cleavage in tissue culture in the presence of up to 1 nM thrombin (Schuepbach et al. 2008). Thus, a low rate of PAR₁ cleavage is not necessary for the generation of protective downstream signaling. Thrombin-cleaved PAR₁ is rapidly internalized and degraded (Shapiro and Coughlin 1998), whereas APC-activated PAR₁ remains on the cell surface and accumulates upon prolonged incubation even when thrombin is present (Schuepbach et al. 2008). Although it remains to be established that the surface-retained cleaved PAR₁ indeed mediates protective signaling, it is interesting to note that both barrier protective signaling and surface retention of APC-cleaved PAR₁ were detected up to low nanomolar thrombin concentrations. Downstream G-protein coupling of PAR₁ can differ for specific agonists (McLaughlin et al. 2005b). Therefore it is conceivable that even though thrombin and APC activate PAR₁ by cleaving the same scissile bond (Kuliopulos et al. 1999), differences in the interaction of the tethered ligand with PAR₁ could lead to the activation of distinct downstream signaling pathways and to unique biological responses, including distinct trafficking of thrombin- and APC-cleaved PAR₁. The finding that cellular trafficking of thrombin- and APC-cleaved PAR₁ is distinct suggests how receptor signaling by a very inefficient protease can be relevant in the presence of the much stronger agonist thrombin. Because of the irreversibility of proteolytic activation, PAR₁ signaling must be regulated through mechanisms such as receptor trafficking (Shapiro and Coughlin 1998; Trejo 2003). Although the rate of thrombin-PAR₁ cleavage at any given point in time may be much higher than the rate of APC-PAR₁ cleavage, the thrombin-cleaved receptor is rapidly internalized and degraded whereas the APC-cleaved receptor accumulates on the surface and can potentially mediate relevant signaling in the presence of thrombin (Fig. 4.2). This illustrates how the efficiency of induction of a specific biological response does not necessarily correlate with efficiency of cleavage.

4.10 Conclusion

Inflammatory and thrombotic responses are intimately connected through various interactions. The finding that the same receptor PAR₁ mediates both thrombin and APC signaling demonstrates an unexpected level of integration in the regulation of vascular physiology. Several novel concepts may help explain how using a single

receptor cell can sense proteolytic activity of thrombin and APC independently. Although much remains to be characterized and extensive research will be required to elucidate molecular details of the signaling pathways, these results provide conceptually novel insight into the paradoxical condition that the two key coagulation proteases thrombin and APC, linked by a negative feedback loop, can mediate opposite effects on endothelial biology through the same receptor PAR₁. A better mechanistic understanding of how cells sense the proteolytic activity of thrombin and APC in their microenvironment and how they respond may eventually lead to novel approaches to treat patients with sepsis and other disorders where the inflammatory response plays a key role, including myocardial infarction and stroke.

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Chapter 5

The Role of Thrombin in Vascular Development

Martin Moser and Cam Patterson

Abstract Vasculogenesis is essential for embryonic development. The vasculature and the intravascular blood compartment develop in a close spatial and temporal relationship. Here we discuss how thrombin, as the common final effector of the blood coagulation system, helps to coordinate vasculogenesis. Mouse models lacking coagulation factors result in impaired thrombin generation and display a phenotype of disturbed vasculogenesis. Notably, either impaired thrombin binding to its cellular receptor PAR₁ or disrupted downstream signaling via G-proteins results in very similar phenotypes in mouse models. Given that vasculogenesis in adults follows comparable signaling patterns as vasculogenesis in embryos, understanding these pathways allows the possibility of identifying potential therapeutic targets for the use in the treatment of cardiovascular disease.

5.1 Introduction

The formation of the cardiovascular system, together with the circulating blood compartment, is a critical step in embryonic development. For normal embryonic development, a close interaction between the two partners is essential. It is now evident that the blood coagulation system, and particularly its common final agonist thrombin, serves as a critical regulator in vascular development. Here we discuss the evidence that supports a role for the coagulation system in general, and thrombin in particular, in normal vasculogenesis. In this chapter, the term *vasculogenesis* is used to describe all blood vessel formation during embryonic development, including the formation of vascular precursor cells and the sprouting events occurring at midgestation. In contrast, *angiogenesis* is the term used to describe sprouting of adult blood vessels from preexisting blood vessels, a topic that is discussed elsewhere in this book.

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5.2 The Coagulation Cascade

The coagulation cascade is described elsewhere in this book in detail. For this chapter we assume a simplified model as depicted in Fig. 5.1. The *extrinsic pathway* is initiated by tissue factor (TF) and activated factor VII (FVIIa), which form a complex that activates factor X (FX). Alternatively, FX can be activated by a catalytic complex formed by the *intrinsic pathway*. This complex, which is assembled on an appropriate phospholipid surface, is composed of the serine protease FIXa and its cofactor FVIIIa. Once activated, FXa comes together with the nonenzymatic cofactor FVa to form a macromolecular catalytic prothrombinase complex. This complex also assembles on procoagulant phospholipid surfaces, such as activated platelets or inflammatory cells adhering to the site of vascular damage. Finally, the prothrombinase complex converts prothrombin to thrombin. Thrombin, as the common final enzyme of the coagulation cascade, can either act on circulating fibrinogen to convert it to fibrin, or interact with cell surface receptors to induce intracellular pathways.

Over the past decade *in vitro* studies as well as *in vivo* loss-of-function models have contributed to the elucidation of the mechanisms involved in vasculogenesis. Today, the classical components of the coagulation cascade are well established; however, the mechanisms by which these same molecules participate in developmental events within the vascular system are not intuitive. Although there are numerous *in vitro* experiments addressing the role of thrombin in vasculogenesis,

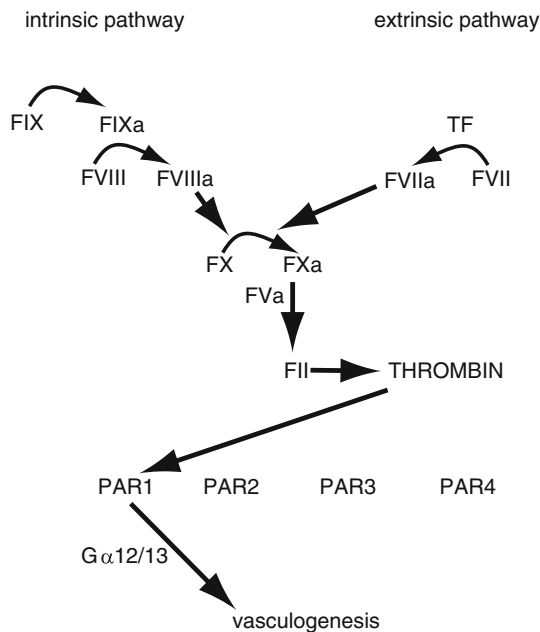


Fig. 5.1 Simplified scheme of the coagulation cascade

in this chapter we will focus on the *in vivo* experiments. If the coagulation cascade and/or its final common effector thrombin are involved in vasculogenesis *in vivo*, then impaired thrombin generation should result in altered blood vessel formation in the embryo proper as well as in the yolk sac. In several notable models this has proven to be the case (Table 5.1). We will consider this evidence in detail here.

5.3 Intrinsic Pathway

Because the production of thrombin is not entirely dependent on the intrinsic pathway, loss-of-function mutations involving the enzymes associated with this pathway do not result in drastic phenotypes. For example, postnatal bleeding disorders are the major feature reported in mouse models of FIX or XIII deletion (Lin et al. 1997). Likewise, in humans the genetic impairment of FVIII or FIX causes hemophilia A or B respectively. FVIII or FIX mutations in neither mice nor humans result in vascular defects. However, because thrombin generation is not entirely dependent on the intrinsic pathway, the lack of vascular defects associated with impaired FVIII or FIX function does not necessarily discount a central role for thrombin in embryonic vasculogenesis.

5.4 Extrinsic Pathway

5.4.1 *Tissue Factor*

Tissue factor is the initiator of the extrinsic pathway. Under physiologic conditions, TF is a cell surface protein that is restricted to subendothelial layers of the blood vessel wall. However, when blood coagulation is needed quickly (e.g., after vascular injury or capillary leakage), or upon endothelial cell activation (such as in inflammation, septic shock, or within tumor vasculature), TF becomes exposed to the vessel lumen. Subsequently, TF acts as an indispensable cofactor for the activation of FX by FVII.

The critical importance of TF in vasculogenesis is clearly demonstrated in mouse models of TF deficiency. The loss of TF in mouse models results in embryonic death of 80–100% (depending on the genetic background) of the TF^{-/-} embryos at around embryonic day (ED) 10.5 (Bugge et al. 1996; Carmeliet et al. 1996; Toomey et al. 1996). TF-deficient embryos are pale and suffer from massive hemorrhaging from embryonic and extraembryonic vessels. The large vitelline vessels are missing in these animals, and the yolk sac capillaries are dysmorphic and fuse to form a disordered plexus. Residual blood flow is detectable within the embryo proper (which is generally less affected) but not in the yolk sac vasculature. In other studies involving transgenic mice in which the native form of TF was replaced by various mutations of human TF, it was demonstrated that only the extracellular

Table 5.1 Mouse models of coagulation factor deficiencies

Mutation	Pathway	Embryonic lethality (%)	ED	Phenotype	Reference
FIX	Intrinsic	No		Spontaneous bleeding after birth	Lin et al. 1997
FVIII	Intrinsic	No		Extensive bleeding after injury	Bi et al. 1995
Tissue factor	Extrinsic	80–100	8.5–10.5	Bleeding and vascular defects in embryonic and extraembryonic tissues	Bugge et al. 1996; Carmeliet et al. 1996; Toomey et al. 1996
FVII	Extrinsic	No	–	Bleeding after birth; death within 3 weeks	Rosen et al. 1997
FX	Common	~30	11.5–12.5	Bleeding, no vascular defects detected, high resorption rate	Dewerchin et al. 2000
FV	Common	~50	9.5–10.5	Bleeding and vascular defects in yolk sac	Cui et al. 1996
FII	Common	~50	10.5	Bleeding, vascular defects in one study	Sun et al. 1998; Xue et al. 1998
TM	Receptor	Complete	~8.5	Developmental retardation, no red blood cells	Healy et al. 1995
PAR ₁	Receptor	~50	~9.5	Pericardial effusion, small vascular defects	Connolly et al. 1996; Darrow et al. 1996
Gα ₁₃	Signaling	Complete, endothelial: 50	~10.5	Vascular defect in yolk sac and embryo proper	Offermanns et al. 1997; Ruppel et al. 2005
FV and PAR ₁	Combined	~96	~12.5	Bleeding in embryonic and extraembryonic tissues	Griffin et al. 2001

ED embryonic day

domain, containing the FVII binding capacity, is necessary for embryonic survival (Parry and Mackman 2000). A deletion mutant of the cytoplasmic domain of TF can still rescue embryonic lethality whereas expression of mutants with dysfunctional extracellular domains that are unable to bind FVII cannot overcome lethality in *TF*^{-/-} embryos.

These studies demonstrate that TF is necessary for proper embryonic development; lack of TF, and presumably the subsequent lack of active thrombin, results in characteristic vascular defects. The FVII binding capacity is the essential feature of TF in embryonic vasculogenesis, whereas the induction of intracellular signals by the cytoplasmic domain appears not to be vital. Taken together, these studies indicate that the initiation of the extrinsic pathway of blood coagulation is a prerequisite for normal vascular development and subsequently embryonic survival.

5.4.2 Factor VII

FVII is the key soluble ligand for TF, and the complex formed by TF and FVII activates FX. Given the severe vascular defects resulting from *TF* deletion, it is somewhat surprising that *FVII*-deficient embryos develop to term normally (Rosen et al. 1997). During embryonic development, *FVII*^{-/-} mice do not display vascular defects; however, as neonates these mice die from severe bleeding within the first days after birth. This suggests that although embryonic FVII is not necessary for embryonic development, survival after birth is critically dependent on this factor. Given the tight interaction between FVII and TF, how can it be then that *TF*-deficient animals die during embryonic development while *FVII*-deficient animals survive till after birth? These apparently contradictory results can be explained as follows.

First, there may be placental leakage of maternal FVII replacing the lack of embryonic FVII in *FVII*-deficient mice. In intracardiac blood samples from *FVII*^{-/-} embryos, the FVII procoagulant activity is less than 0.05% of adult levels (Rosen et al. 1997). Additionally, in transfer experiments less than 0.1% of maternally injected recombinant FVII is detected in the embryo, indicating that although FVII is unlikely to cross the placental barrier in major amounts, transfer of small, but perhaps physiologically relevant amounts of FVII cannot not be excluded. Interestingly, small amounts of other coagulation factors (TF, FV) have been demonstrated to rescue the phenotypes of their respective null mice (Parry and Mackman 2000; Yang et al. 2000). Consequently, it is very well possible that small amounts of FVII below the limits of detection still maintain thrombin signaling and proper vasculogenesis in *FVII*-deficient embryos.

An alternate hypothesis as to why *FVII*-deficient mice survive to birth is that while FVII is the main TF ligand, other proteins may be able to compensate in its absence. Efforts have been made to address this possibility by culturing wild-type embryos with rNAPc2, a nematode protein that specifically inhibits the TF/FVII complex. Unfortunately the results of these studies were inconclusive, leaving open the possibility that FVII is not the only TF ligand and that other proteins may

compensate for FVII loss or inactivation (Rosen et al. 1997). Thus, although the differences in the phenotypes of the FVII and TF null mice are not entirely resolved, because maternal transfer or bypass pathways cannot be excluded, the extrinsic pathway does appear to be essential for proper vasculogenesis.

5.5 Common Pathway

FX is the first protein of the common pathway of the coagulation cascade. It can be activated by either the extrinsic or intrinsic pathways and in turn cleaves prothrombin using nonenzymatic FV as a cofactor. Approximately one third of all FX^{-/-} mouse embryos die between ED 11.5 and 12.5 (Dewerchin et al. 2000). In studies looking at these mutants, a thorough histological analysis of all embryos was not possible because of a large degree of embryonic resorption. However, of the few embryos that could be studied, bleeding was evident, although a direct determination of vascular defects was not possible. Nevertheless, it is clear that the FX^{-/-} genotype causes embryonic lethality, and hemorrhage may be the most obvious cause. Since FX and FV act together, it is of particular interest to compare the phenotypes of FV^{-/-} and FX^{-/-} mice (Cui et al. 1996). In contrast to divergent phenotypes seen in mutants of the molecular couple TF and FVII, deletion of FV has consequences similar to those observed in FX^{-/-} mice. An FV^{-/-} genotype results in 50% embryonic lethality at ED 9.5–10.5. Although the surviving embryos continue to develop to term, neonates die immediately after birth because of massive hemorrhaging. Additionally, a vascular phenotype with abnormalities in the yolk sac vasculature as well as a reduced number of blood islands is evident in FV^{-/-} mice. Efforts have been undertaken to address questions about temporal and spatial effects of FV expression; however, these studies were not able to yield satisfying answers, most likely because of technical issues related to the respective experimental design (Yang et al. 2000). Regardless, the similar midembryonic lethality of up to 50% seen in both FX^{-/-} and FV^{-/-} mice is consistent with a model where impaired thrombin generation results in defective vasculogenesis.

5.6 FII

The assembly of the prothrombinase complex results in cleavage of prothrombin to active thrombin. Two independent groups have studied FII gene-disruption. In both reports, embryonic lethality was about 50% at ED 10.5 (Sun et al. 1998). In the first study, the surviving pups died within days of birth from severe hemorrhage, although an abnormal vascular phenotype was not detected in the FII-deficient pups. In the second study, most embryos surviving beyond ED 10.5 developed to ED 14.5, but almost all embryos died by ED 18.5 (Xue et al. 1998). In this study, embryos that survived the first critical stage at ED 10.5 had characteristic defects in yolk sac vasculature with enlarged capillaries and capillary fusion into a venous

plexus. The rare neonates that did make it to birth were pale and died of hemorrhage soon after. Although the two reports detailing the phenotype of FII-deficient animals share the feature of incomplete embryonic lethality, it is interesting to note that only one study reports the occurrence of vascular defects. This inconsistency could be explained by the different genetic backgrounds of the mouse models. The latter model, in which vascular defects were noted, involved FII-deficient mice on a C57BL/6J background. This study also reported that the degree of late embryonic lethality was higher. These results could suggest that the FIIa deficiency is more pronounced on a pure genetic background (such as C57BL/6J), possibly because of a smaller degree of compensation that may be found when similar mutations are bred into mice on a mixed genetic background.

In summary, the common pathway of the coagulation cascade is essential for thrombin generation, and the available data support the notion that thrombin, as the final common effector of the coagulation cascade, is important in embryonic vasculogenesis and survival. Mouse models of disrupted common pathway coagulation factors result in midembryonic lethality of about 50%, notably in the case of FX and in one study of FII. Nevertheless, it is noteworthy that lethality does not exceed 50% in many of the respective animal models, which indicates that other molecules or pathways can at least partially compensate for the loss of common pathway factors during vascular development.

5.7 Blood Coagulation and Vasculogenesis

The studies described above clearly indicate that mutations in the reported genes associated with the plasma coagulation cascade result in neonatal bleeding and death and, by extension, that sufficient plasma coagulation is critical for survival after birth. However, some of these mutations are also accompanied by bleeding during embryonic development, especially in mutants with vascular abnormalities. The ontogenetic question then is, what relationship exists between vascular abnormalities and bleeding in these mice? Does bleeding occur in these embryos because of a lack of sufficient coagulation? Or is it a direct result of vascular abnormalities? The answers to these questions can be found by comparing mouse models in which clotting defects are caused by factors *downstream* of thrombin activation. For example, mice lacking the transcription factor NF-E2 lack circulating platelets (Shivdasani et al. 1995). These mice die from hemorrhage, indicating that their clotting system is indeed defective. However, embryonic development is not altered in these animals and embryonic bleeding is also not reported. Consequently, insufficient platelet activation as a result of low thrombin levels does not necessarily explain embryonic bleeding in coagulation-factor-deficient mice. Along the same lines, mice lacking the fibrinogen alpha chain, which is required for effective thrombus formation, do not display a vascular phenotype, nor do they bleed during embryonic development (Suh et al. 1995). Thus, both downstream events of thrombin activation (i.e., platelet activation and fibrinogen cleavage) do not seem important in controlling embryonic bleeding. This supports the notion that vascular malformation

and physical disintegrity may be the main cause for embryonic bleeding. Indeed, embryonic bleeding has also been reported in mouse models of disturbed vascular signaling that result in a vascular phenotype completely unrelated to blood coagulation such as *Tie1*^{-/-} and *Tie2*^{-/-} mice (Dumont et al. 1994).

It is possible that each of the coagulation factors (TF, FX, FV, FII, and maybe FVII) has a distinct influence on vascular development. However, since the common final step of the coagulation cascade is the generation of active thrombin, a more likely assumption is that thrombin itself is the critical factor in terms of homeostatic blood vessel formation. However, the fact that vascular abnormalities are not reported in all coagulation-factor-deficient mouse models does seem to contradict this assumption. This apparent contradiction could be explained by the fact that, in some cases, it is technically extremely challenging to assess vascular abnormalities and endothelial discontinuations. Likewise, many mutant embryos halted from proper development undergo quick resorption and are therefore frequently not accessible for further examination. As a consequence, it is very well possible that embryonic lethality in *FV* and *FII*-deficient mice is related to a vascular phenotype during development even though these phenotypes cannot be easily visualized. Another question plaguing the hypothesis that thrombin is necessary for proper blood vessel formation is, why do some coagulation factor mutants suffer from embryonic lethality while others (such as *FVII*, *FVIII*, and *FIX* mutants) do not? For the intrinsic pathway proteins *FVIII* and *FIX* this is most likely because small but physiologically relevant amounts of thrombin can still be produced even when the intrinsic pathway is completely blocked. In the case of *FVII* the situation is most likely different. The extrinsic pathway is necessary for thrombin generation, therefore *FVII*^{-/-} mice should not be able to produce even small amounts of thrombin and should therefore die in utero. The fact that they do not suggests that another source of *FVII* must be available. As discussed above, maternal transfer of *FVII* is the most likely source of *FVII* in these mice. Such a phenomenon has recently been described for *FX*-mutant mice (Tai et al. 2008) and may also occur for other soluble coagulation factors such as *FIX* and *FII*.

In summary, the data obtained by analyzing animal models with impaired coagulation factors clearly suggest that thrombin is the critical protein involved in vasculogenesis. However, knowing that thrombin does not exert its effects via downstream events of the coagulation cascade, such as platelet activation and fibrin generation, leaves open the possibility that thrombin binding to its receptors plays an important role in this process.

5.8 Thrombin Receptors

There are at least two types of thrombin receptors, thrombomodulin (TM) and the protease-activated receptors (PARs), both of which are described in detail in other chapters of this book.

TM is a transmembrane receptor (Esmon 1987). When thrombin binds to TM, thrombin's procoagulant activity is lost, and instead, the natural anticoagulant protein

C is activated (Fuentes-Prior et al. 2000). This results in the formation of a negative feedback loop that results in the cleavage of active FV and FVIII and, consequently, less procoagulant activity. Disruption of the TM gene in mice results in embryonic lethality at two different stages. The first block occurs prior to significant expression of thrombin, suggesting a role for TM which is independent from thrombin (Healy et al. 1995). Selective expression of the extracellular domain of TM rescues the developmental block, but the second stage of embryonic lethality still occurs at midgestation from consumptive coagulopathy (Isermann et al. 2001). These results suggest that TM plays an important role in placental development; however, a direct link between TM and vasculogenesis is not obvious.

In contrast, PARs (the second set of thrombin receptors) do play an important role in vasculogenesis as evidenced by lack of function models. Disruption of the gene for PAR₁ results in 50% embryonic lethality (Connolly et al. 1996; Darrow et al. 1996). A cause of embryonic death has not been determined definitively but it is noteworthy that failed hemostasis is not present. Detailed analyses of PAR₁^{-/-} mice revealed that small openings develop in the sinus venosus that appear large enough to allow red blood cell passage. Interestingly, PAR₁ expression under the control of an endothelial-cell-specific promoter is able to increase survival of PAR₁^{-/-} mice to more than 80%, indicating that endothelial expression of PAR₁ is crucial to embryonic survival. PAR₁^{-/-} mice suffer from embryonic lethality at a comparable stage of development, as is seen in coagulation-factor-deficient mice. The activity of the plasma coagulation cascade is not altered in these mice, consistent with the fact that thrombin's proteolytic activity is not related to the presence or absence of PARs. These observations suggest that thrombin must have an influence on embryonic lethality and vasculogenesis by binding to its PAR₁ receptor.

As PARs are G-protein-coupled receptors, additional insight into thrombin's role in vascular development may be derived by considering the phenotype of G-protein-deficient mice. Gα₁₃ is a PAR₁-interacting protein that is required for intracellular thrombin signaling. Disruption of the Gα₁₃ gene results in 100% embryonic lethality in homozygous mice between ED 9.5 and 10.5 (Offermanns et al. 1997), presumably from the complete lack of vascular structures and only occasional blood islands seen in these animals. To address the question of whether endothelial cell signaling is essential in this context, endothelial-cell-specific Gα₁₃^{-/-} mice have been generated. Indeed, these mice are a virtual phenocopy of the PAR₁^{-/-} mice (Ruppel et al. 2005). Thus, the phenotypes observed in models of defective *thrombin generation* are in accordance with those observed in models for impaired *thrombin binding*. It is likely that these phenomena occur along the same thrombin-signaling pathway.

5.9 Diversity of Thrombin Signaling

Signaling within the coagulation cascade is much more diverse than a simplified model of the coagulation cascade might suggest. For example, by using genetically modified mice, Griffin et al. (2001) tested whether FV mediates conversion of

prothrombin to thrombin, which in turn then signals via PAR₁. Embryonic lethality for FV^{-/-} mice and PAR₁^{-/-} mice are very similar (about 50%), although lethality of FV^{-/-} mice (Cui et al. 1996) tends to occur slightly later than reported for PAR₁^{-/-} mice (Connolly et al. 1996). Embryonic phenotypes between these mouse models are similar although not identical. If a simple signaling model is true, then intercrosses of FV^{-/-} and PAR₁^{-/-} mice should produce a phenotype identical to that of either deficiency alone (Griffin et al. 2001). However, only 4% of the FV^{-/-}, PAR₁^{-/-} embryos survive to term. The dead embryos display a more severe vascular phenotype than either of the single gene mutants alone, suggesting that the FV and PAR₁ pathways may interact, although probably do not overlap completely. Assuming that no maternal FV crosses to the deficient embryo, this would mean that other agonists could still activate PAR₁ even in the absence of embryonic FV. This also implies either that molecules other than FV can activate prothrombin to thrombin, or that PAR₁ is activated by other proteases in addition to thrombin. Indeed, Riewald and coworkers (2001) have shown that in the absence of thrombin, FXa has the capacity to cleave PAR₁ and to induce the same downstream signals induced by thrombin cleavage of PAR₁. Activated protein C has also recently been shown to activate PAR₁ (Yang et al. 2007). Consequently, under certain circumstances, prothrombin-dependent activation of PAR₁ can be bypassed, consistent with the partial embryonic lethality seen in FII^{-/-} mice. In the surviving mice, these alternative pathways must be sufficient to compensate for the loss of prothrombin.

The search for FV-dependent signaling molecules continues. Interestingly, it has been demonstrated that PAR₂ can be activated by FVII in vascular endothelial cells in a FX- and TF-dependent manner (Camerer et al. 2000). This effect seems to be independent of thrombin and may contribute to continued FV-dependent endothelial cell signaling even in the absence of PAR₁. Recently, it has been shown that the interaction between thrombin and PAR₁ is not restricted to a direct interaction alone. Thrombin binding to TM induces protein C activation, which in turn may activate PARs (Riewald et al. 2002). This might serve as an example of the complexity of molecular interactions that are involved in thrombin signaling. These extensive interactions and the resulting parallel options for particular pathways are supported by the genetic data, as only TF^{-/-} and Gα₁₃^{-/-} mice undergo complete embryonic lethality because of defects in vasculogenesis. For the other coagulation factor nullmouse models, compensating pathways rescue at least a cohort of the embryos.

5.10 Conclusion

Several lines of evidence demonstrate that thrombin signaling is essential for vasculogenesis. Thrombin's vasculogenic activity is independent from its coagulant activity and depends mostly on signaling via the thrombin receptors. Under certain conditions, not only thrombin but also other molecules (FVII, FX) are able to activate these receptors and induce downstream signals. Lack of *thrombin generation* (as seen in TF^{-/-}, FX^{-/-}, FV^{-/-}, FII^{-/-} mice) results in severe vascular defects in

embryonic development. Notably, similar phenotypes occur in models of impaired *thrombin binding* to its PAR receptor (PAR₁^{-/-} mice) or in a model missing the corresponding G-protein in endothelial cells (Gα₁₃^{-/-} mice). It is striking that TF^{-/-} embryos suffer from complete lethality at midgestation in contrast to other coagulation factor or PAR deficiencies. This phenomenon might indicate that TF is an essential initiator of the extrinsic pathway. In contrast, the loss of other proteins involved in this pathway might be compensated for by other family members or by the induction of different pathway interactions.

The use of coagulation cascade proteins to link the fluid blood component with vessel wall development may be an important means to control the close temporal and spatial connection of these two components of the cardiovascular system. It may be that gaps in the developing vasculature are detected by TF exposure to the bloodstream and that through its subsequent activation of the coagulation cascade, a signal is created to seal off the leaking vessel by endothelial differentiation and growth. It is noteworthy that although there is an increasing understanding of the signaling mechanisms described in this chapter, the particular molecules and events that link thrombin signaling to vasculogenesis are not yet known completely. Our understanding of the molecular mechanisms involved in signaling required for vasculogenesis is far from complete, but by close analysis of the molecular pathways involved it might be possible in the future to create molecular tools to manipulate vasculogenesis in human disease.

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Chapter 6

The Role of Thrombin in Angiogenesis

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Abstract The suggestion of the late Judah Folkman that “solid tumors are angiogenesis-dependent” in the 1970s stimulated a multidisciplinary research effort to understand the complex cascade of events involved in new blood vessel formation under physiological and pathological conditions. A plethora of endogenous modulators of angiogenesis has been identified, and their roles in the molecular and cellular events that mediate and regulate angiogenesis have been proposed. In addition, it has been recognized that besides solid tumors a large number of common diseases such as ocular diseases, inflammation, etc., have as underlying pathology the derangement of angiogenesis. This prompted a major effort of the biotechnology industry to identify targets and develop agents for the so called *angiogenesis-based therapies*. A brief overview of the regulation of angiogenesis and the clinical applications that have resulted thus far is presented. Furthermore, our finding that thrombin is a potent angiogenic mediator that may play a pivotal role in orchestrating angiogenic factors led us to summarize the recent findings on the role of the coagulation cascade and its components in angiogenesis. Thrombin is a promoter of angiogenesis by activating PAR₁ receptors in platelet and endothelial cells. This identifies PAR₁ as a target for inhibiting angiogenesis with potential therapeutic applications. In addition, thrombin plays a role in promoting angiogenesis by PAR₁-independent mechanisms. Through its RGD sequence, thrombin serves as an adhesive and aptotactic factor for endothelial cells. Thrombin is a potent antiapoptotic factor for endothelial cells, pointing to a novel role of thrombin in vascular protection and integrity. The implications of these findings in the overall regulation of angiogenesis and their possible significance in pathological states are discussed.

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6.1 Angiogenesis in Health and Disease

Angiogenesis is a fundamental physiological process, both in development and in the adult stage of the organisms. The early blood vessels of the embryo and yolk sac in mammals develop by aggregation of de-novo-forming angioblasts into a primitive vascular plexus (vasculogenesis), which then undergoes a complex remodeling process, in which growth, migration, spouting, and pruning lead to the development of a functional circulatory system (angiogenesis) (Coultas et al. 2005). After birth, angiogenesis continues to contribute to organ growth. In adulthood, most blood vessels remain quiescent and angiogenesis occurs only in limited situations such as in the cycling ovary and in the placenta during pregnancy. However, endothelial cells retain their remarkable ability of dividing rapidly in response to a physiological stimulus, such as hypoxia. As such, angiogenesis is reactivated during wound healing and repair (Eming et al. 2007).

The complexity and the realization that derangement of angiogenesis is evident in various common diseases have attracted enormous interest over the past two decades. The potential exists for therapeutic interventions in pathological conditions where an imbalance in the growth of blood vessels contributes to the pathogenesis of numerous disorders (Carmeliet 2005). Angiogenesis has been implicated in more than 70 disorders so far, and the list is ever growing. In disease states such as cancer, ocular and inflammatory disorders, the angiogenic stimulus becomes excessive and the balance between stimulators and inhibitors is tilted, resulting in an angiogenesis switch. Many tumors promote their own growth and dispersion to form metastases by recruiting blood vessels to grow into the vicinity of the tumor (so-called tumor angiogenesis) (Folkman 2006). In other diseases, such as ischemic heart disease, the angiogenic switch is insufficient, causing endothelial cell dysfunction, vessel malformation, or regression or preventing revascularization, healing, and regeneration.

A multidisciplinary research from studies in physiology, developmental biology, mouse genetics, cell and molecular biology, and experimental pathology and drug development resulted in understanding many of the cellular and molecular events that mediate and regulate blood vessel formation. However, the precise control mechanisms involved in a given organ or pathological state are not clearly understood. Until recently, it was generally accepted that in adults the formation of new blood vessels occurs through sprouting of capillaries from existing blood vessels and depends exclusively from the proliferation and migration of homing fully differentiated endothelial cells (Risau 1997). Recent studies have shown, however, that circulating bone marrow-derived endothelial progenitor cells (EPCs) are incorporated into sites of neovascularization and differentiate into mature endothelial cells (Asahara and Kawamoto 2004). This suggests that incorporation of EPCs into the lumen contributes to the growing vessels, thus complementing resident endothelial cells in sprouting new vessels. Since the identification of this cell population by Asahara et al. (1997) several studies have shown reduced numbers and/or impaired function of EPCs in a variety of cardiovascular risk states. On the other hand,

cardiovascular protective factors are known to increase EPC number and function (Kopp et al. 2006). In addition to their role in the maintenance of vascular integrity, EPCs are thought to participate in the process of tumor vascularization through mobilization from the bone marrow and recruitment to sites of tumor vessel formation (Dome et al. 2007).

Angiogenesis is highly complex and requires a dynamic, temporally and spatially coordinated action of a variety of soluble angiogenic mediators, cell-adhesion molecules, and extracellular matrix (ECM) proteins on endothelial and mural cells. Interactions between endothelial cells and vascular mural cells (perivascular cells/vascular smooth muscle cells) have recently come into focus as central processes in the regulation of vascular formation, stabilization, remodeling, and function (Bergers and Song 2005). Failure of the interaction between the two cell types, as seen in different genetic mouse models, results in severe and often lethal cardiovascular defects. In addition, several other cell types are potentially involved. For example, immediately after injury and during wound healing angiogenesis, different subsets of leukocytes are attracted to the wound site, which release a complex array of mediators, which trigger, sustain, and potentially terminate the angiogenic response (Eming et al. 2007).

In recent years, a plethora of mediators, both positive and negative, have been identified, which are crucial for vessel formation, and new insight has been gained into their specific interaction in controlling vascular remodeling and angiogenesis. Vascular endothelial growth factor-A (VEGF-A) and its receptors are the best characterized signaling pathway, which has emerged as a key pathway both in embryonic vascular development and physiological and pathological angiogenesis in the adult (Ferrara 2004). VEGF-A binds to two receptor tyrosine kinases, VEGFR-1 (Flt-1) and VEGFR-2 (KDR, Flk-1). Of the two it is now generally agreed that VEGFR-2 is the major mediator of the mitogenic, angiogenic, and permeability-enhancing effects of VEGF-A. Other crucial signaling molecules that have an established role in the development and differentiation of the vessel wall include platelet-derived growth factor-BB and its receptor PDGFR- β (Lindhal et al. 1997) and the angiopoietins (Angs), the ligands of the Tie2 receptor (Yancopoulos et al. 2000). PDGF-B is required for recruitment of pericytes and maturation of the microvasculature. Ang-1 is generally accepted as the major agonist for Tie2 and is required for further remodeling and maturation of the initially immature vasculature. On the contrary, Ang-2 may act as an antagonist or a partial agonist for Tie2 (Maisonpierre et al. 1997). However, more recent evidence indicates that, unexpectedly, Ang-2 has a positive role, at least in tumor angiogenesis (Oliner et al. 2004).

On the other hand, angiogenesis seems to be under the control of negative regulatory factors. Although several potential negative regulators of angiogenesis have been identified, relatively little is known about their role in the physiological regulation of angiogenesis, and their precise mechanisms of action remain to be more clearly defined (Nyberg et al. 2005). Thrombospondin and several fragments of larger proteins have been described as endogenous inhibitors of angiogenesis including endostatin, tumstatin, and vasostatin. The most recently described

endogenous inhibitor of angiogenesis is vasohibin, which seems to be derived from the endothelium and to operate as a feedback regulator (Watanabe et al. 2004).

As in many physiological and pathological processes of tissue remodeling, ECM molecules play a pivotal role in angiogenesis (Darland and D'Amore 1999). Basement membranes provide essential information for the organization and orientation of endothelial cells and are crucial for the proper function of blood vessels. It is known that removal of some of the major components of the basement membrane leads to leakiness of the vessels (Poschl et al. 2004), and the inhibition of basement membrane biosynthesis blocks angiogenesis (Maragoudakis et al. 1993). During neoangiogenesis, new vessels sprout out of existing vessels and grow along the growth factor gradient. To initiate this process, endothelial cells must degrade the basement membrane and the surrounding ECM with the help of matrix metalloproteases (MMPs, Roy et al. 2006). The contribution of MMPs in angiogenesis, especially MMP-2, MMP-9, and membrane-type metalloproteinase-1 (MT1-MMP), has been convincingly established by the use of natural or synthetic MMP inhibitors, both *in vitro* and *in vivo*. Genetic studies, using mice deficient in those endopeptidases, showed reduced angiogenic responses (Itoh et al. 1998; Vu et al. 1998; Zhou et al. 2000).

Integrins provide the physical interaction with the ECM necessary for cell adhesion, migration, and positioning. They also induce signaling events essential for cell survival, proliferation, and differentiation. Among the integrins expressed in endothelial cells, integrins $\alpha 5 \beta 1$ and $\alpha v \beta 3$ have been proposed to be required for embryonic vascular development and postnatal angiogenesis (Ruegg and Mariotti 2003). Interestingly, integrins dynamically participate in a network with soluble molecules and their receptors (Serini et al. 2008). Several integrins regulate the effect of growth factors (e.g., VEGF, angiopoietins, PDGF) on endothelial cells through interaction with their tyrosine kinase receptors. Moreover, pro- and antiangiogenic factors can directly bind integrins and regulate endothelial cell behavior. In preclinical studies, pharmacologic inhibition of integrin function efficiently suppressed angiogenesis and inhibited tumor progression. These encouraging results led researchers and industry to design pharmacological inhibitors of integrin function for clinical development (Stupp and Ruegg 2007).

Despite some initial setbacks and negative clinical trial results, significant progress has been made over the past few years in targeting angiogenesis for human therapies (Ferrara and Kerbel 2005). In February 2004, the US Food and Drug Administration (FDA) approved bevacizumab, a humanized anti-VEGF-A monoclonal antibody, for the treatment of metastatic colorectal cancer in combination with 5-fluorouracil, after successful phase III studies showing a survival benefit with combined therapy (Hurwitz et al. 2004). In December 2004, the FDA approved pegaptinib, an aptamer that blocks the 165 amino-acid isoform of VEGF-A, for the treatment of the wet (neovascular) form of age-related macular degeneration (Gragoudas et al. 2004). These achievements have validated the notion that angiogenesis is an important target for cancer therapy and other angiogenesis-related diseases. They also have provided a number of important insights and raised various outstanding issues including those related to side effects (Carmeliet 2005;

Ferrara and Kerbel 2005; Verheul and Pinedo 2007). Furthermore, the hope that *therapeutic angiogenesis* will provide a treatment for ischemic disorders still remains unfulfilled, in spite of considerable preclinical and clinical efforts. Over the past decade, intensive efforts have been undertaken to develop therapeutic strategies to promote revascularization of ischemic tissues. Unfortunately, clinical trials testing some proangiogenic factors did not yield the expected results, indicating that the growth of durable and functional vessels is a more formidable challenge than previously anticipated (Simons 2005). Novel strategies, involving transplantation of bone-marrow-derived cells or the delivery of molecules or vectors capable of stimulating the growth not only of distal capillaries but also of proximal collateral conduit vessels, may be required in the future.

6.2 Angiogenesis and the Coagulation System

Activation of clotting, vascular thrombosis, and deposition of extracellular fibrin are early steps in the angiogenic response induced by tumors, wound healing, and inflammation (Rickles et al. 2003; Dvorak 2005). Injury triggers a complex array of enzymatic reactions resulting in the activation of coagulation cascade and hemostasis (Eming et al. 2007). Coagulation constitutes and hemostatic plug provide the basic stimulus to initiate the inflammatory response, angiogenesis, and tissue formation. Similarly, components of the coagulation system contribute to cancer biology (Wojtukiewicz et al. 2004; see chapter by Kobrinisky and Karparkin in this volume). The mechanism by which coagulation is activated in cancer is multifunctional. Tissue factor (TF) has been recognized to play an important role in this process (Daubie et al. 2007). TF is aberrantly expressed in many tumor cell types, and increased TF expression in tumors is associated with increased angiogenesis and higher tumor grades (Swada et al. 1999; Ueno et al. 2000; Nakasaki et al. 2002; Guan et al. 2002). TF-induced angiogenesis may be due to the upregulation of VEGF and downregulation of thrombospondin (Zhang et al. 1994; Abe et al. 1999). TF was also demonstrated to mediate angiogenesis through activation of its cytoplasmic domain. Phosphorylation of TF-cytoplasmic domain results in cell migration and protease-activated receptors (PARs) signaling (Versteeg and Ruf 2006). The activation of PAR₁ and PAR₂ by either TF/FVIIa complex or the TF/FVIIa/FXa complex lead to an acceleration of angiogenesis (Belting et al. 2004). The currently available information on the multiple effects of the TF pathway on tumor pathophysiology and angiogenesis provides the basis for considering TF as a target for antitumor and antiangiogenic treatment. Indeed, tissue factor pathway inhibitor (TFPI), the naturally occurring TF inhibitor, has been shown to exhibit antitumor effects in vitro and in vivo (Amirkhosravi et al. 2007). The TFPI inhibits angiogenesis in the chick embryo model and significantly reduces melanoma, colon, and lung carcinoma-induced angiogenesis (Fernandez et al. 2004). In addition, TFPI-2, a structural homologue of TFPI, which inhibits the TF/FVIIa complex, has been shown to function in the maintenance of the stability of the tumor environment

and inhibits invasiveness and growth of neoplasms, as well as metastases formation (Sierko et al. 2007). TFPI-2 was also been shown to induce apoptosis and inhibit angiogenesis in experimental models (Yanamandra et al. 2005; Ivanciu et al. 2007). Of interest, TFPI-2 in endothelial cells is upregulated by VEGF and suppresses proliferation of endothelial cells (Xu et al. 2006). This may represent a mechanism for negative-feedback regulation of VEGF activity.

Activated protein C (APC) and protein C inhibitor (PCI) are the major components of the anticoagulant protein C pathway. Recently, APC and PCI have been demonstrated to play many roles not only in the regulation of hemostasis but also in cell inflammation, proliferation, apoptosis, tumor biology, and angiogenesis (Suzuki and Hasyashi 2007; see chapter by Riewald in this volume). Regarding angiogenesis, it was recently reported that APC increases proliferation of vascular endothelial cells and angiogenesis by APC receptor-mediated activation of mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase, and endothelial nitric oxide synthase (eNOS) pathways (Uchiba et al. 2004). On the contrary, PCI was shown to inhibit the growth and metastatic potential of breast cancer cells and angiogenesis *in vivo* and *in vitro* through a mechanism independent of its protease inhibitory activity (Asanuma et al. 2007).

As mentioned in the previous section, a variety of endogenous angiogenesis inhibitors have been described that are derived from the proteolytic processing of parent proteins with distinct action. Among them, the generation of antiangiogenic forms of antithrombin (O'Reilly et al. 1999) and prothrombin kringle-2 (Lee et al. 1998) provides additional evidence of a more general process in which components of the clotting system play major role in the regulation of angiogenesis (Browder et al. 2000). Cleavage of the carboxyl-terminal loop of antithrombin induces a conformational change in the molecule, and the cleaved conformation has potent antiangiogenic and antitumor activity in mouse models (O'Reilly 2007). In this regard, prothrombin kringle-2 domain also exhibits antiproliferative activity in endothelial cells (Lee et al. 1998). Furthermore, recombinant human prothrombin kringle-1, 2 have potent antiangiogenic activities in chick embryo angiogenesis model and inhibit Lewis lung carcinoma tumor growth and metastasis in mice (Kim et al. 2002).

Interestingly, thrombin, the final common effector of coagulation cascade, has also been found to have important roles in angiogenesis (Tsopanoglou and Maragoudakis 2004). Thrombin is a central multifunctional molecule that can interact with a wide range of proteins that regulate its production and activity in a different fashion (see chapter by Di Cera and Gruber in this volume). It acts as a procoagulant when it converts circulating fibrinogen into an insoluble clot that anchors platelets to the site of lesion. Thrombin stabilizes the ensuing clot by activation of factor XIII and enhances its own generation from prothrombin by promoting TF production by endothelial cells and activation of factors V, VIII, and XI. Thrombin-mediated PARs signaling triggers platelet activation and aggregation and unfolds the prothrombotic role of thrombin in the blood. On the other hand, thrombin acts as an anticoagulant when it activates protein C. Thrombin generated locally in the vicinity of the intact endothelium binds to the cell surface glycoprotein thrombomodulin, which redirects thrombin specificity from fibrinogen and PARs to the

zymogen protein C. APC downregulates the further progression of the coagulation cascade. Binding of thrombomodulin also favors thrombin's irreversible inactivation by antithrombin, which scavenges thrombin, using heparin as a cofactor and limits its life in the blood to only a few minutes. Thrombin also upregulates TFPI-2 synthesis, which inhibits TF pathway activity and in turn downregulates thrombin generation (Neaud et al. 2004).

The angiogenesis-promoting effect of thrombin was first demonstrated in the chick chorioallantoic membrane system (Tsopanoglou et al. 1993). Thrombin also promoted the formation of blood vessels in matrigel plug injected subcutaneously into mice (Haralabopoulos et al. 1997). In these *in vivo* systems, it was shown that the angiogenic action of thrombin is dose-dependent and requires that the catalytic site of thrombin be functional, since the D-Phe-Pro-Arg-chloromethylketone-thrombin (PPACK-thrombin, chemically inactivated analog of thrombin at the active site) is without effect and competes with thrombin for its angiogenic action. An analog of thrombin (γ -thrombin), which is catalytically active, but lacks the anion-binding exosite for binding fibrinogen and therefore cannot form fibrin, is also active in promoting angiogenesis. In addition, thrombin receptor activating peptide SFLLRN, which acts as an agonist peptide for activating PAR₁, is also effective in activating angiogenesis. These findings led us to conclude that the angiogenic action of thrombin can be receptor-mediated and independent of fibrin formation and therefore it can be modulated without interfering with blood coagulation. In fact, it is now recognized that thrombin influences angiogenesis through both coagulation-dependent and coagulation-independent mechanisms. The coagulation-dependent pathway involves platelet activation and fibrin formation. Coagulation-independent mechanisms involve PAR₁-mediated signaling activation in endothelial and other vascular cells.

6.3 Thrombin-Induced Angiogenesis: Involvement of Coagulation-Dependent Pathways

Vessel wall injury or thrombus formation stimulates platelets to adhere to subendothelial matrix and to undergo activation by thrombin, leading to aggregation and degranulation. Platelets stimulate endothelial cell proliferation and tube formation *in vitro* and induce angiogenesis *in vivo* (Pipili-Synetos et al. 1998; Verheul et al. 2000). The absence of platelets inhibits the early stages of angiogenesis and contributes to the formation of decreased number of new vessels *in vivo* (Rhee et al. 2004; Kisucka et al. 2006). It should be emphasized that platelet progenitor cells (megakaryocytes) synthesize and secrete VEGF, whereas mature platelets transport and, upon activation by thrombin, release this growth factor (Mohle et al. 1997; Verheul et al. 1997; Wartiovaara et al. 1998). Moreover, platelet α -granules are the source of a many of other proangiogenic factors, including VEGF-C (Wartiovaara et al. 1998), basic fibroblast growth factor (bFGF or FGF-2) (Friesel and Maciag 1999), and PDGF (Guo et al. 2003). On the other hand, apart from being

proangiogenic, platelet α -granules are the source of inhibitors of angiogenesis, such as thrombospondin (Good et al. 1990), Ang-1 (Pizurki et al. 2003), and endostatin (Ma et al. 2005). It is of interest that thrombin, which influences platelet activity through platelet PAR_1 and PAR_4 receptors, triggers VEGF secretion via PAR_1 activation whereas thrombin activation of PAR_4 leads to the release of endostatin (Ma et al. 2005). Activated platelets are also the source of microvesicles circulating in the bloodstream (Freyssinet 2003), which have been documented to induce angiogenesis both in vitro and in vivo (Brill et al. 2005; Janowska-Wieczorek et al. 2005).

Despite its rather large molecular weight, the plasma protein fibrinogen is capable of leaking into the extravascular tissue. During wound healing, inflammation or malignant tumor growth, fibrinogen then binds to specific receptors on inflammatory and tumor cells and is cleaved by thrombin generated in the local microenvironment (Rickles et al. 2003; Laurens et al. 2006). Several reports provide evidences that this fibrin network has a supportive role for endothelial cell adhesion and angiogenesis. Fibrin-containing chambers, which were implanted subcutaneously in guinea pigs, induced an angiogenic response within 4 days. Vessels were formed and entered the chambers through surface pores (Dvorak et al. 1987; Liu et al. 1990). The fibrin matrix also appears as an excellent substrate for the invasion of endothelial cells and subsequent formation of new capillary-like structures (Koolwijk et al. 1996). Fibrin bridges cell-matrix interactions essential for physiologic and pathologic events, which is accomplished through exposure of cryptic sites in the molecule that facilitate adhesion to cell-surface receptors (Medved et al. 2001). For example, binding of endothelial cells to fibrin via the adhesion molecule vascular endothelial cadherin may be necessary for capillary tube formation (Martinez et al. 2001). Endothelial cells express different adhesion molecules on their surface based on the extracellular matrices they encounter. Fibrin matrix provokes an angiogenic response by upregulating the expression of $\alpha v \beta 3$ receptors that facilitate endothelial invasion and capillary tube formation (Clark et al. 1996; Dallabrida et al. 2000). The $\alpha v \beta 3$ integrins provide survival signals to endothelial cells during their interaction with fibrin.

The fibrin matrix also provides storage of proangiogenic growth factors, such as bFGF, VEGF, and insulin-like growth factor-1. Within the fibrin, sequestered growth factors are protected from proteolytic degradation (Sahni et al. 2000). Degradation of the matrix by proteolytic enzymes, generated during invasion by endothelial and/or tumor cells, releases sequestered growth factors, which bind to cognate receptors on the invading cells, promoting cell proliferation and migration for tumor angiogenesis (Sahni et al. 1999; Sahni and Francis 2000). Moreover, fibrin E-fragment, which is produced by proteolytic cleavage of fibrin, has been shown to stimulate angiogenesis in the chick chorioallantoic membrane assay (Thompson et al. 1992).

Similarly, it is known that thrombin is also trapped within fibrin matrix and protected from inactivation by its circulating inhibitors. Binding of thrombin to the fibrin or subendothelial ECM leaves the majority of the molecule functional and available for cellular interaction (Bar-Shavit et al. 1989). Indeed, thrombin has been proposed as a novel ligand of $\alpha v \beta 3$ and $\alpha 5 \beta 1$ integrins (Tsopanoglou et al. 2002; Papaconstantinou et al. 2005). When endothelial cells are cultured on thrombin-coated surfaces, the interaction between thrombin and cellular integrins facilitates their

attachment and migration and protects them from apoptosis. These effects of thrombin are independent of its catalytic action and PAR₁ activation and involve the single RGD (Arg-187, Gly-188, Asp-189) sequence within the thrombin molecule (Tsopanoglou et al. 2004). DIP-thrombin, an active site chemically inhibited analog, or the catalytically inactive thrombin mutant S195A, which replaces the active site serine with alanine, are equally effective in promoting cell attachment and migration. According to the crystal structure of thrombin, most of the RGD sequence is buried under the 220- and 186-loops and is not available for interactions with integrins (see chapter by Di Cera and Gruber in this volume). Unlike bound thrombin on solid surfaces, thrombin in solution requires the integrity of its catalytic activity in order to produce its cellular effects. In addition, when thrombin is in solution each of the three residues of the RGD sequence affects, in a specific way, the allosteric machinery of thrombin and therefore impinges on the ability of the enzyme to cleave PARs and elicit cellular responses (Fig. 6.1). More specifically, it was found that the extent of the PAR₁-mediated cellular responses (e.g., intracellular calcium mobilization) to RGD-mutants, and to many other thrombin mutants, correlates well with their ability to bind Na⁺ and transduce this event into enhanced catalytic activity (unpublished data). However, when thrombin is immobilized, it can assume a noncanonical conformation exposing the RGD sequence to the solvent and allowing functioning as an epitope, which is recognized by specific integrins that mediate cellular signaling without the involvement of the catalytic activity of the enzyme (Fig. 6.1) (Papaconstantinou et al. 2005).

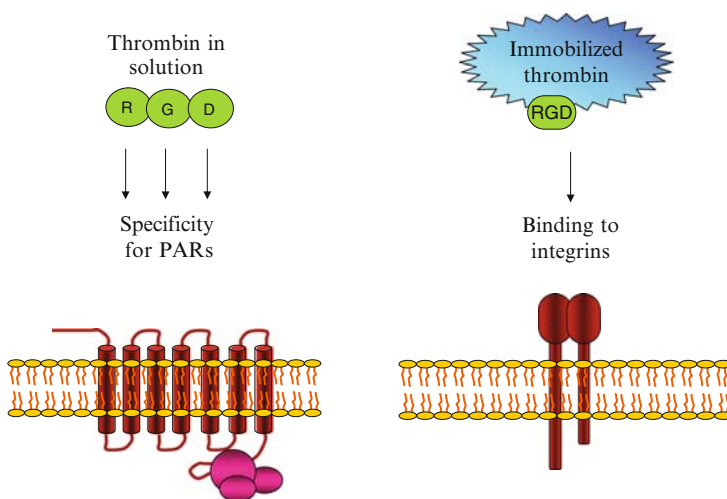


Fig. 6.1 Dual role of RGD sequence within thrombin. When thrombin is in solution Arg187, Gly187, and Asp189 are implicated in the allosteric regulation of the enzyme by Na⁺, which controls the specificity of the enzyme toward protease-activated receptors (PARs). When thrombin is immobilized on a solid surface, it assumes a different conformation, probably because it is subject to constraints and forces not seen in solution, with the RGD sequence exposed and available for interactions with integrins

The aforementioned effects of thrombin are most likely contributing to initiation of angiogenesis, providing a plausible explanation for the angiogenesis *par excellence* occurring within thrombi in several pathophysiological conditions. For example, a very common clinical observation is that after thrombosis in large vein, the thrombus is recanalized by new vessels seen with angiography. Interestingly, recent data provide evidence that thrombin bound to fibrin clot confers angiogenic and haemostatic properties to EPCs, which have been shown to be involved in recanalizing venous thrombi (Smadja et al. 2008).

6.4 Thrombin-Induced Angiogenesis: Involvement of Coagulation-Independent Mechanisms

Apart from its role in platelets activation and fibrin generation, thrombin exerts a wide range of effects on endothelial cells, which may contribute to the control of many functions, including vascular tone, hemostasis, inflammation, and angiogenesis (Martorell et al. 2008). Thrombin, mainly through PAR₁ signaling, stimulates endothelial cells and regulates the release, expression, and activation of the majority of angiogenesis mediators. Thrombin-induced angiogenesis in a chick CAM system is associated with upregulation of VEGF as well as Ang-2 (Caunt et al. 2003). In line with this, thrombin upregulates VEGF (Huang et al. 2001) and Ang-2 (Huang et al. 2002) in endothelial cells. Another important effect of thrombin is the potentiation of mitogenic activity of VEGF on endothelial cells (Tsopanoglou and Maragoudakis 1999). When endothelial cells are preincubated with thrombin and subsequently exposed to VEGF, the mitogenic activity is increased more than 100% over that expected from the additive effects of thrombin and VEGF alone. This synergistic effect of thrombin with VEGF can be explained by the finding that thrombin significantly increases mRNA levels and functional receptor protein for the VEGFR-2. Thus, the upregulation of VEGF receptor by thrombin sensitizes endothelial cells to the action of VEGF for the activation of angiogenesis. In this context, it was recently demonstrated that thrombin markedly upregulates growth-regulated oncogene- α in endothelial cells, and this chemokine in turn mediates the thrombin-induced increase of vascular regulatory growth factors (VEGF, Ang-2) and receptors (VEGFR-2) (Caunt et al. 2006). Furthermore, different studies have reported that thrombin upregulates the hypoxia-inducible factor 1 α (HIF-1 α) under nonhypoxic conditions by a reactive oxygen species (ROS)-dependent mechanism both in endothelial cells (Dupuy et al. 2003) and vascular smooth muscle cells (Gorlach et al. 2001; BelAiba et al. 2004). Thrombin has also been shown to activate the proliferation of endothelial cells by acting directly as mitogenic factor (Olivot et al. 2001). This effect of thrombin involves the phosphorylation of extracellular signal-regulated protein kinase 1/2 (Erk1/2, MAPK) and is mediated by epidermal growth factor (EGF) receptor transactivation through MMP-dependent release of heparin-binding EGF (Zania et al. 2008). Also, neuron-derived orphan receptor-1, a nuclear receptor, has been shown to mediate thrombin-induced endothelial cell mitogenesis and migration (Martorell et al. 2007).

It has been shown also that thrombin alters endothelial cell function via PAR₁ signaling by decreasing endothelial cell ability to adhere to ECM proteins (Tsopanoglou and Maragoudakis 1998). This action of thrombin together with its ability to activate the MMP-2 in a PAR₁-independent manner (Zucker et al. 1995, Fernandez-Patron et al. 1999) may be of great importance at the initial stages of angiogenesis, when endothelial cells must detach from their anchorage sites of the vessel wall, dissolve the surrounding basement membrane, migrate to distal sites, proliferate, and form the lumen of new vessels. It may also be important in this respect that thrombin increases the levels of mRNA and protein of $\beta 3$ integrin subunit in endothelial cells (Tsopanoglou et al. 2002). As a result, endothelial cells exposed to thrombin have increased ability to interact with proteins of the ECM such as vitronectin and fibronectin. Integrin $\alpha v\beta 3$ in the surface of endothelial cells recognizes the RGD sequence present in proteins of the ECM. Interaction of the RGD sequence with endothelial cell $\alpha v\beta 3$ integrin regulates the attachment, migration, growth, and apoptosis of these cells.

In addition to modulating the preexisting endothelial cells, thrombin may also impact repair mechanisms and angiogenesis by affecting bone marrow-derived progenitor cell. It was recently shown that human EPCs as well as CD34+ cells expressed the thrombin receptor PAR₁ at their surface, at levels similar to those found on mature endothelial cells (Smadja et al. 2005). Thrombin, through PAR₁, acts as a potent inducer of bone marrow-derived cell proliferation, migration, and differentiation into endothelial cells (Tarzami et al. 2005), by an angiopoietin-dependent mechanism (Smadja et al. 2006). Furthermore, thrombin inhibits apoptosis and causes proliferation of vascular progenitor cells, expressing both markers for activated endothelial cells and vascular smooth muscle cells, suggesting a significant role of thrombin in regenerative repair by circulating progenitor cells (Chen et al. 2008).

6.5 Thrombin Is a Protection Factor for Endothelial Cells

A growing body of evidence has accumulated showing that thrombin is pro- or antiapoptotic in several cell types, including epithelial and neuronal cells, fibroblasts, and tumor cells (Flynn and Buret 2004). In these cells, activation of PAR₁ has been shown to induce or inhibit apoptosis, depending on thrombin concentration or that of PAR₁ agonist peptides. In contrast to these observations, it was found recently that thrombin protects endothelial cells from apoptosis via a mechanism in which its catalytic active site and PAR₁ activation have limited contribution (Zania et al. 2008). PAR₁ activation contributes only approximately 20% on the total thrombin antiapoptotic effect. This protective effect of thrombin may be of importance for the migrating endothelial cells during angiogenesis. A further demonstration of the distinct mechanism of thrombin-induced cell survival was obtained from experiments with DIP-thrombin, a chemically inactivated thrombin analog at the active site. DIP-thrombin is mimicking the antiapoptotic effect in endothelial cells almost to the same extent as thrombin itself. In addition, it was shown that $\alpha v\beta 3$

and $\alpha 5\beta 1$ integrins play an essential role in the activation of cell survival by thrombin. When echistatin, which is a very potent antagonist of $\beta 3$ - and $\beta 1$ -integrin families, or neutralizing monoclonal antibodies against $\alpha v\beta 3$ and $\alpha 5\beta 1$ are combined with thrombin, its protective effect is almost abolished. Collectively, these findings suggest that thrombin inhibits apoptosis in endothelial cells by at least two mechanisms: a minor contribution is mediated by PAR_1 activation and a major contribution by interaction with $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins in which the catalytic site of thrombin is not necessary.

The involvement of thrombin in endothelial cell survival may open new insights on the role of thrombin in vascular protection and provides evidence for an essential contribution of thrombin in the establishment and maintenance of vessel wall integrity. Vascular protection can be considered as a distinct nonangiogenic process through which thrombin can enhance endothelial cell functions that lead to inhibition of vascular smooth muscle cell proliferation, endothelial cell survival, and suppression of thrombotic and inflammatory events. When thrombin binds to its receptor thrombomodulin on the surface of endothelial cells it loses its procoagulant functions and generates APC, which provide negative feedback regulation of thrombin generation. In addition, a number of studies have indicated that APC can also regulate several cellular functions of endothelial cells, including survival (Esmon 2006; see chapter by Riewald in this volume). Intravenous infusion of recombinant APC reduces mortality in patients with severe sepsis (Van de Wouwer et al. 2004). Current evidence indicates that APC has profound anti-inflammatory effects, which at least in part are independent from its role as an anticoagulant. Also, thrombin increases endothelial expression of complement inhibitory proteins (e.g., decay accelerating factor) (Lidington et al. 2000) and enhances the production and release of nitric oxide (NO) and prostacyclin I_2 (PGI_2) in endothelial cells (Macfarlane et al. 2001). An important function of these two intercellular mediators is vasodilation, but NO and PGI_2 also have several other effects that may play vascular protective roles, including the inhibition of endothelial and smooth muscle cell proliferation (Gang and Hassid 1989; Shirotani et al. 1991; Heller et al. 1999), antiplatelet action (Whittle et al. 1978; Schmidt and Walter 1994), antiapoptotic effect (Liou et al. 2006), and inhibition of leukocyte adhesion (Kubes et al. 1991). Therefore, it can be proposed that in addition to being angiogenic, thrombin also acts as a vascular protective factor via maintenance of antiapoptotic pathways and stimulation of APC, NO, and PGI_2 production by endothelial cells. Vascular protection may provide an attractive alternative mechanistic framework for understanding the impact of thrombin on the cardiovascular system.

Interplay between proangiogenic growth factors and vascular protection mechanisms is essential for the cascade of reactions involved in blood vessel formation and maturation. In this context, the thrombin-mediated arterial protection together with its potent angiogenic ability may prove to be useful in the treatment of occlusive and ischemic cardiovascular diseases. The formation of functional collateral vessels with vascular stability is essential for the improvement of distal tissue ischemia (Hershey et al. 2001). In fact, therapeutic angiogenesis aimed at improving blood flow by stimulating the growth and development of collateral vessels has

been proposed as a potential treatment for ischemic cardiovascular diseases (Vale et al. 2001). The strategy was based on bio-bypassing the underperfused tissues and has been attempted using various angiogenic growth factors (Idris et al. 2004). Despite numerous investigations, the challenge remains in identifying the factor or the combination of factors that will stimulate functional neovascularization.

We have found recently that a single administration of thrombin is capable of establishing functional vascular networks in a rabbit hind-limb ischemic model (unpublished data). Computerized quantification of the vessel area and length of large collaterals ($d > 500\mu\text{m}$), which is visualized by digital subtraction angiography, provides a statistically significant increase in thrombin-treated limbs, as compared with control limbs. Similarly, the functional estimation of the blood flow distal to the excised femoral artery shows a significant improvement in the limbs that have received thrombin. In line with these results, De Paula et al. (2006) showed that the use of anticoagulant drugs immediately after induction of tissue ischemia hampers spontaneous angiogenic response in a rodent hind limb ischemia model. Furthermore, the ability of thrombin to contribute to the formation of functional vessels was also evidenced in chick chorioallantoic membrane vascular corrosion casting system (Dimitropoulou et al. 2002). This study shows that thrombin significantly increases not only the number of vessels in CAM but also their diameters and lengths. In conclusion, these findings provide evidence that thrombin through its multiplicity of effects on angiogenesis, survival, interaction with other growth factors and many cell types may have the unique ability to orchestrate the requirements for the development of mature blood vessels.

6.6 Thrombin and PAR_1 as Targets for Inhibiting Angiogenesis

Increased body of evidence coming from clinical trials suggests that adjunctive therapy with anticoagulants may improve prognosis in cancer patients (see chapter by Petralia and Kakkar in this volume). Both heparins and vitamin K antagonists have been tested in this context. The rationale for an antitumor effect of anticoagulant drugs may rely on their capacity to inhibit blood coagulation. The inhibition of coagulation may affect cancer progression mainly by the reduction of thrombin generation and subsequent platelet activation and fibrin formation, which are highly elicited by the tumor itself and favor tumor growth and metastasis. As mentioned previously, thrombin is a cellular signaling protease, which acts a potent proangiogenic factor for endothelial cells. In addition, fibrin plays several roles in tumor progression, i.e., induces the expression of TF and angiogenic cytokine IL-8 by endothelial cells, supports the migration of tumor cells out of the vasculature, and provides a scaffold to the formation of new blood vessels (Falanga et al. 2003). In line with these findings, the cell-penetrating pepducin P1pal-7, which acts as potent PAR_1 antagonist, significantly blocks tumor growth and angiogenesis of breast cancer xenografts in nude mice (Boire et al. 2005). Recently, two newly developed PAR_1 antagonists, SCH79797 and RWJ56110, have been evaluated for their effects

in the angiogenic cascade (Zania et al. 2006). Using the in vivo model of the chick chorioallantoic membrane system of angiogenesis, it was shown that SCH79797 and RWJ56110 are very potent antiangiogenic agents. This inhibitory effect is dose-dependent and is evident both for basic angiogenesis and that stimulated by thrombin. PAR₁ antagonists also inhibit capillary-like structure formation by endothelial cells cultured either in a medium containing serum or the combination of bFGF and VEGF. Furthermore, the antiangiogenic effect of PAR₁ antagonists is well correlated with their inhibitory effects on endothelial cell growth. These agents not only arrest endothelial cell proliferation and prevent vessel growth, but also induce regression of existing vessels by increasing endothelial cell apoptotic death. It is of interest that the inhibitory effect of PAR₁ antagonists was evident only when endothelial cells were at a fast-growing state. Together these results provide further evidence that thrombin and its receptor, PAR₁ are key molecules that mediate angiogenesis and validate the concept that inhibitors of these targets would be effective antiangiogenic agents and as such have the potential therapeutic application in cancer and other angiogenesis-related diseases.

6.7 Conclusion

This chapter points to the vast complexity of the angiogenic cascade, even when one considers the involvement of the components of the coagulation process. This is expected for such a vital biological phenomenon, which needs to be activated at a moments notice in every tissue most likely in a unique way, in order to produce blood vessels of different architectural and physiologic characteristics. The interplay of the plethora of modulators of angiogenesis and their relative temporal and spatial formation are unlikely to be suitable for targeting a single factor for modulation of angiogenesis. Perhaps this explains the relative slow progress of translating the large body of knowledge on angiogenesis to therapeutic applications. The multiplicity of actions of thrombin in angiogenesis and its interrelation with so many other key players in the angiogenic process may qualify thrombin as a good candidate for orchestrating the overall process.

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Chapter 7

Thrombin and Thrombin Peptides in Wound Healing and Tissue Repair

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Abstract Thrombin and thrombin peptides play a key role in wound healing and tissue regeneration. Early events initiated by thrombin contribute to inflammatory cell recruitment and activation of inflammatory cells. Certain nonproteolytic effects of thrombin, or thrombin peptides presumably released from fibrin clots, also appear to affect revascularization and progression of the repair process. Thus, the role of thrombin in wound healing goes far beyond hemostasis. Recent animal studies and human clinical trials with TP508, a specific 23 amino acid peptide representing a receptor-binding domain of thrombin, show significant improvement in healing and revascularization of dermal wounds and bone fractures. These studies highlight a role of thrombin peptides in wound healing that is just beginning to be recognized.

7.1 Introduction

Wound healing is typically divided into four phases based on the order in which they occur: hemostasis, inflammation, proliferation, and remodeling. A key initiator of the hemostatic process is the activation of prothrombin to its proteolytic form, thrombin. The role of thrombin in initiating hemostasis through platelet activation and fibrin clot formation is well understood. The second inflammatory phase begins immediately after hemostasis with recruitment of inflammatory cells (neutrophils and monocytes) and their activation to release cytokines and chemokines, which in turn recruit additional inflammatory cells and progenitor cells to the wound site. Thrombin appears to play a key role in this early inflammatory phase, stimulating chemotaxis and production of inflammatory cytokines and chemokines. As wounds proceed into what has been called the proliferative phase, angiogenesis and proliferation of precursor cells takes place followed by cellular differentiation and

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tissue remodeling. The potential role of thrombin in these later stages of healing is less well understood.

The major focus of this review is to explore the complex nature of the involvement of thrombin and thrombin peptides in both the inflammatory phase of wound healing and in later stages of this process. Many early cellular events are stimulated by proteolytically active thrombin working through proteolytically activated receptors (PAR₁, PAR₃, or PAR₄) as reviewed by Ramachandran et al. in this volume. It appears that other cellular events, however, can be stimulated by proteolytically inactive thrombin molecules and thrombin peptides released from the clot. These proteolytically inactive thrombin-derived molecules also stimulate early recruitment and activation of inflammatory cells, but in addition appear to attenuate the inflammatory response and promote later stages of wound healing. Thus thrombin and its peptides appear to be involved in virtually all phases of tissue repair.

In this chapter we will (1) review data demonstrating that cellular signaling occurs by both proteolytic and nonproteolytic cellular interactions of thrombin, (2) place these results into a conceptual model for the temporal interactions that orchestrate tissue repair, and (3) review recent animal and human clinical studies in which synthetic thrombin peptides have been used to better understand the role of thrombin-derived molecules in wound healing and tissue repair.

7.2 Proteolytic and Nonproteolytic Cell Signaling

The concept of thrombin involvement in postclotting wound healing began with the discovery that thrombin stimulated fibroblast proliferation (Chen and Buchanan 1975; Zetter et al. 1976; Carney et al. 1978), and that this stimulation involved specific high-affinity cell-surface receptors (Carney and Cunningham 1978). ¹²⁵I-thrombin binding to mouse embryo and hamster lung fibroblasts produced linear Scatchard plots suggesting a single class of high-affinity receptors with an apparent K_d of 2–4 nM and with ~150,000 binding sites per cell (Carney and Cunningham 1978; Van Obberghen-Schilling and Pouyssegur 1985). These thrombin receptors appeared to cluster on the surface of cells (Carney 1983), but were not rapidly internalized through receptor-mediated internalization (Bergmann and Carney 1982; Carney and Bergmann 1982; Carney 1983; Van Obberghen-Schilling and Pouyssegur 1985). Thrombin receptors involved in cellular activation were subsequently identified on neutrophils (Sonne 1988) and endothelial cells (Shimada and Ozawa 1985; Belloni et al. 1987). These findings demonstrated that thrombin has specific high-affinity receptors on several cell types that are involved in inflammation and tissue repair.

To better understand the signaling events initiated by thrombin, various thrombin derivatives were tested for their ability to bind to thrombin receptors and to stimulate cell proliferation. Thrombin inactivated by diisopropyl fluorophosphate

(DIP-thrombin) was shown to bind to thrombin receptors with an affinity equivalent to that of α -thrombin (Glenn et al. 1980; Van Obberghen-Schilling and Pouyssegur 1985), yet proteolytically active gamma-thrombin (γ -thrombin) did not compete with DIP-thrombin or α -thrombin for receptor binding (Glenn et al. 1980). Interestingly, stimulation of fibroblast proliferation involved both a nonproteolytic receptor occupancy-dependent signal (that could be generated by DIP-thrombin) and a separate proteolytic signal that was stimulated by γ -thrombin, even though γ -thrombin did not appear to bind to thrombin high-affinity receptors (Carney et al. 1984, 1986b). Notably, binding and signal generation by the two different derivatives appeared to be noncompetitive. This suggested that two different binding sites or two different receptor molecules were involved in these thrombin interactions. That two different thrombin receptor molecules may be involved in thrombin stimulation of fibroblasts was supported by receptor cross-linking studies. One study (Carney et al. 1979) showed specific crosslinking of proteolytically active thrombin to a receptor molecule of ~55,000 kDa [a size similar to that of proteolytically activated receptors (PAR₁₋₄)]. Two other studies using nonproteolytic thrombin derivatives demonstrated specific binding to a larger molecule of nearly 150,000 kDa (Moss et al. 1983; Van Obberghen-Schilling and Pouyssegur 1985). Signaling by proteolytic and nonproteolytic thrombin derivatives was also reported to be different, further suggesting that two different types of receptors are involved. For example, proteolytic γ -thrombin or α -thrombin signals stimulated phosphoinositide turnover, increased calcium mobilization (Carney et al. 1985), and could be mimicked by activation of protein kinase C (PKC) (Gordon and Carney 1986). In contrast, receptor occupancy by proteolytically inhibited thrombin did not stimulate phosphoinositide turnover (Carney et al. 1985, 1986a, b), but did generate receptor occupancy signals that could be mimicked by a monoclonal antibody selected for its ability to compete with thrombin for cell surface binding (Frost et al. 1987).

In some cases it appears that both proteolytic and nonproteolytic thrombin interactions can have similar effects on cells even though these interactions may initiate different signals. For example, both γ -thrombin (that retains proteolytic activity) and proteolytically inhibited thrombin were shown to be chemotactic for human and sheep neutrophils (Bizios et al. 1986). In addition, endothelial permeability could be stimulated by proteolytically active α -thrombin and γ -thrombin as well as by two different proteolytically inhibited thrombin molecules (Garcia et al. 1986). Thus, both types of thrombin interaction stimulate these cellular events.

The identification and cloning of seven transmembrane domain receptors for thrombin that required proteolytic cleavage for their activation offered an explanation for how proteolytically active thrombin generated signals (see chapter by Ramachandran et al. in this volume). Since proteolytically inactivated thrombin derivatives such as those described earlier, however, have no proteolytic activity, it was hypothesized that nonproteolytic signals were generated by a high-affinity interaction of thrombin with a separate receptor. This putative nonproteolytically

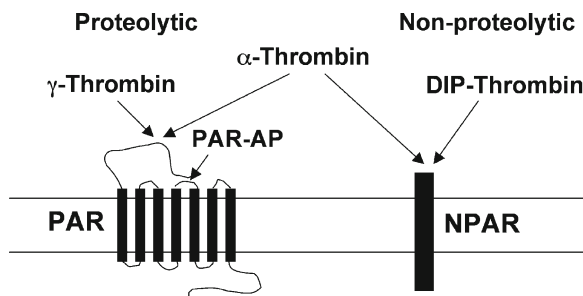


Fig. 7.1 Working model reflecting two types of thrombin interaction: proteolytic activation of proteolytically activated receptors (PAR) by α -thrombin, γ -thrombin, or PAR-activating peptides (PAR-AP), and the nonproteolytic activation of a nonproteolytically activated receptor (NPAR) by α -thrombin or DIP-thrombin. See text for details

activated receptor has been referred to as NPAR to distinguish it from the PAR receptors. Figure 7.1 represents a model for separate PAR and NPAR receptors for thrombin. This model provides a conceptual basis for experiments to determine the nature of NPAR. It is possible, for example, that NPAR represents one of the molecules known to interact with thrombin (e.g., integrins, thrombomodulin, protease nexin), that it represents a new thrombin receptor, or that NPAR represents an as-yet-to-be-defined nonproteolytic interaction with one of the PAR receptors.

7.3 Biological Activity of Thrombin Peptides

To define the region of thrombin responsible for high-affinity binding to fibroblasts a series of peptides representing various regions of the thrombin molecule were synthesized and tested for their capacity to compete with thrombin for binding to fibroblasts (Glenn et al. 1988). One of these peptides, TP0508, representing prothrombin amino acids 508–530 (AGYKPDEGKRGDACEGDSGGPFV, CAS #497221-38-2) was shown to compete with thrombin for its binding to thrombin receptors. The peptide had very little mitogenic activity by itself, but was shown to stimulate proliferation of cells when added in combination with proteolytically active γ -thrombin, submitogenic concentrations of α -thrombin, or PMA to activate PKC (Carney et al. 1986b; Gordon and Carney 1986). Thus, TP508 appeared to stimulate the same set of receptor-occupancy-dependent mitogenic signals that were stimulated by proteolytically inactivated thrombin derivatives. Other peptides including a thrombin fragment that activated macrophages (Bar-Shavit et al. 1984, 1986) did not compete for thrombin binding to fibroblasts or stimulate proliferative signals (Carney, unpublished). Therefore, the effects of the TP508 peptide appeared to be quite specific and were consistent with TP508 binding to a putative NPAR receptor.

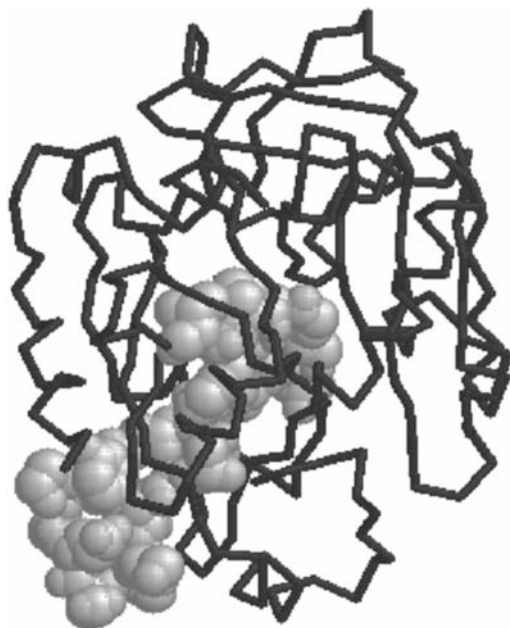


Fig. 7.2 Location of TP508 within the structure of thrombin. TP508 amino acids are depicted as balls within a simplified three-dimensional line structure of thrombin (Papaconstantinou et al. 2005; Di Cera and Gruber this volume)

The region of thrombin represented by TP508 includes an RGD sequence. When one looks at the three-dimensional structure of thrombin, however, the RGD portion of the molecule is located at the bottom of thrombin's proteolytic pocket (Fig. 7.2). The presence of an RGD in the TP508 sequence suggested possible interactions of TP508 and thrombin with integrins. The location of the RGD sequence within thrombin rather than its exposure on the cell surface, however, raised questions about how this thrombin region could interact with cell surface receptors. Two theories evolved to explain how this region might be exposed to interact with receptors on cells.

First was the concept that proteolytic cleavage of thrombin released peptides or partially degraded thrombin molecules that retained biological activity. Thrombin appears to be sequestered selectively within fibrin clots or in the subendothelial matrix where it can retain activity and be protected from inhibition by protease inhibitors (Wilner et al. 1981). A number of studies have indicated, however, that thrombin can be cleaved by plasmin and elastase (Brower et al. 1987; Bar-Shavit et al. 1991), suggesting that upon cleavage thrombin may undergo a conformational change, exposing the RGD site, or that cleavage may release bioactive thrombin peptides. This further suggested that the clot itself could act as a *time clock* to allow controlled release of bioactive peptides as enzymes produced by neutrophils migrating into the wound degrade the clot. Since neutrophils are chemotactically attracted to proteolytically inactive thrombin molecules (Bizios et al. 1986), released thrombin peptides with

similar activity would recruit additional cells to insure that healing proceeded to the centre of the wound and did not stop at the periphery.

Second, studies from laboratories of Bar-Shavit (Bar-Shavit et al. 1991) and Maragoudakis (Tsopanoglou et al. 2002) showed that thrombin immobilized to plastic or subendothelial matrix enhanced binding and spreading of endothelial cells through a mechanism that involved the $\alpha v \beta 3$ integrin binding to RGD regions of thrombin. The TP508 thrombin peptide immobilized to plastic had similar effects (Tsopanoglou et al. 2004). Since the RGD region of thrombin was not on the surface of native thrombin where it could interact with integrins, this suggested that upon thrombin cleavage or its interaction with surfaces, thrombin may undergo a conformational change that would expose this site (Bar-Shavit et al. 1991). Indeed, little cell adhesion was observed with native thrombin, but adhesion was increased greatly by nitration of tyrosine residues (NO_2 - α -thrombin) or preincubation of thrombin at 37°C where autolysis appeared to expose the RGD region (Bar-Shavit et al. 1991). Recent studies confirmed that an alternative structure of thrombin occurs in high salt allowing the RGD sequence to be exposed on the surface of the thrombin molecule (Papaconstantinou et al. 2005). These studies support the concept that thrombin may become conformationally altered in the postclotting wound environment with or without proteolytic cleavage.

It is interesting to note that modification of thrombin by nitration, addition of MeSO_2 , exosite affinity labeling, or thrombin autolysis increases the ability of cells to bind to immobilized thrombin by exposing the RGD region (Bar-Shavit et al. 1991). These same modifications, however, inhibit thrombin binding to its high-affinity receptor (Carney and Cunningham 1978). Thus, there appears to be an inverse structural relationship between thrombin derivative molecule binding to integrins and the binding of these derivatives to high-affinity receptors. This inverse relationship argues that the high-affinity binding of soluble thrombin and thrombin-derived peptides may be distinct from immobilized thrombin interaction with integrins.

If effects of soluble TP508 are not mediated through integrins, one must ask if these peptides act through nonproteolytic interactions with one of the PAR receptors. Two lines of evidence suggest that this is not the case. First, after a PAR receptor is activated by thrombin, the receptors are rapidly internalized through mechanisms that involve their recruitment to clathrin-coated pits (Hoxie et al. 1993). This appears to be in direct conflict with early studies demonstrating that the receptors to which thrombin bound with high affinity were excluded from coated pits and not internalized by receptor-mediated endocytosis (Bergmann and Carney 1982; Carney and Bergmann 1982). Since TP508 competed with thrombin for cell surface binding, these data suggest that TP508 either binds to a molecule separate from PAR receptors or that it interacts with PAR receptors in a manner that activates transmembrane signals without stimulating receptor internalization. Second, studies with radiolabeled TP508 have demonstrated specific binding and photoaffinity cross-linking of ^{125}I -TP508S to three molecules with approximate molecular weights of 195, 145, and 90 kDa on fibroblasts, endothelial cells, and U937 monocytes (Bergmann et al. 2006). Thus, TP508

binding appears to be to molecules larger than PAR receptors. Interestingly, in these labeling experiments, thrombin competes with labeled TP508 for binding with a K_i of ~ 2 nM suggesting that TP508 binds to the same high-affinity thrombin receptor identified in early thrombin binding experiments or to a subset of these receptors (Carney and Cunningham 1978). Further characterization of this receptor remains essential to understanding the role of thrombin and thrombin peptides in cellular activation.

7.4 Cellular Effects of TP508

7.4.1 Chemotaxis

Based on the chemotactic activity of proteolytically inactive thrombin for neutrophils and monocytes (Bizios et al. 1986), it seemed likely that TP508 may also recruit inflammatory cells, endothelial cells, and precursor cells to the site of injury. Indeed, *in vitro* studies showed that TP508 was chemotactic for human neutrophils (Ramakrishnan and Carney 1993; Ryaby et al. 2006), keratinocytes (Sower et al. 1999a), endothelial cells (Norfleet et al. 2000a), and osteoblasts (Li et al. 2005a). In the Ramakrishnan studies, TP508 stimulated neutrophil chemotaxis in trans-well migration assays, while a PAR₁-activating peptide had little if any effect (see also Ryaby et al. 2006). Interestingly, subsequent studies showed that human neutrophils do not express PAR₁ (Jenkins et al. 1995). Therefore, the chemotactic effects of TP508 on these cells could not be attributed to either proteolytic or nonproteolytic activation of PAR₁, yet in early studies proteolytically active thrombin derivatives were shown to stimulate neutrophil chemotaxis (Bizios et al. 1986). In these cells, PAR₄ may provide proteolytic chemotactic signals while TP508 may generate signals through a separate NPAR or by nonproteolytic activation of a PAR receptor other than PAR₁.

7.4.2 Angiogenesis

Endothelial cells appear to be activated both by thrombin and by proteolytically inactivated thrombin fragments such as TP508. As described earlier, thrombin and TP508 immobilized on plastic stimulate endothelial cell attachment and migration (see chapter by Tsopanoglou and Maragoudakis in this volume). Soluble TP508 also provides chemotactic signals for endothelial cells and is active in angiogenesis assays (Norfleet et al. 2000a; Li et al. 2005a; Ryaby et al. 2006). One study compared the effects of TP508 with several PAR-activating peptides using a microvessel angiogenic sprouting assay (Vartanian et al. 2006). Results showed that TP508 stimulated microvessel outgrowth to an extent similar to or greater than VEGF; however, thrombin and PAR agonists appeared to

inhibit or have no effect on vessel outgrowth. Thus, these studies demonstrate that TP508 is a potent angiogenic factor and further suggest that TP508 exerts this effect through a receptor mechanism that is distinct from that of PAR activation.

7.4.3 *Gene Expression*

Several studies have shown that TP508 treatment of cells initiates changes in gene expression that relate to wound healing. For example, differential display polymerase chain reaction (PCR) was used to compare early (30 min) transcriptional changes in patterns of fibroblast gene expression induced by TP508 to those induced by thrombin and the PAR₁-activating peptide SFLLRN (Sower et al. 1999b). These studies showed that TP508 upregulated a number of genes, including annexin V, which were not upregulated by thrombin or SFLLRN. Since annexin V is an inhibitor of PKC, these data suggested that TP508 may attenuate downstream signals that involve PKC activation. Along these lines, additional studies with fibroblasts showed that TP508 inhibited the upregulation of collagenase by thrombin and TNF α (Sower and Carney 1999). Thus, TP508-initiated signals may have a dual function in stimulating certain transcriptional pathways while inhibiting others.

TP508 changes in gene expression include a number of cytokines. For example, TP508 treatment of peripheral blood mononuclear cells (PBMC) upregulated interleukin-1 β and interleukin-2 (Naldini et al. 2004). Similar effects were seen with stimulation of these cells with thrombin or the PAR₁-activating peptide, SFLLRN (Naldini et al. 2002). In these studies, antisense downregulation of PAR₁ inhibited SFLLRN upregulation of IL-1 β , but did not inhibit the upregulation stimulated by TP508. Thus, it appears that stimulation of certain cytokines may be initiated by either the activation of PAR receptors or the TP508 activation of an NPAR-type receptor distinct from PAR₁. Interestingly, if one activates macrophages with LPS to maximally stimulate cytokine production, TP508 inhibits the stimulation (Naldini et al., unpublished). Thus, an important distinction between effects of nonproteolytic peptides and active thrombin may be the ability of nonproteolytic fragments such as TP508 to attenuate proinflammatory signals.

TP508 changes in gene expression appear to also include genes involved with proliferation, differentiation, and survival of cells. For example, studies with cultured rat chondrochondral chondrocytes showed that the differentiation state of cells could affect their responsiveness to TP508 and that TP508 could affect cellular differentiation (Schwartz et al. 2005). In these studies, TP508 stimulated proliferation of growth zone chondrocytes, but not those isolated from the resting zone. In contrast, TP508 increased proteoglycan production in resting zone cells, but not in growth zone cells. These studies suggest that

TP508 increases proliferation in less differentiated cells of the chondrocytic lineage while promoting maintenance of a more hyaline-like chondrocyte at later stages of differentiation. In these chondrocytes, TP508 also prevented apoptosis induced by chelerythrine, tamoxifen, or inorganic phosphate (Zhong et al. 2008). Thus, TP508 appears to promote proliferation and survival of cells. In a separate study, proteomic analysis of rat fracture calluses demonstrated that TP508 treatment caused an increase in expression of antiapoptotic genes and decreased expression of proapoptotic genes (Li et al. 2007). These findings provide an example of how nonproteolytic thrombin peptides may play a role both in the later proliferative and remodeling stages of wound healing.

7.5 Animal Models of Wound Healing

Studies described earlier indicate that both proteolytic and nonproteolytic effects of thrombin stimulate early inflammatory recruitment of PMNs and monocytes, promote endothelial changes that can lead to angiogenesis, and have proliferative effects on cells. In contrast, the capacity to attenuate inflammatory signals, to promote advanced elongation of angiogenic microvessel sprouts, and to protect cells from apoptosis may be more specific to nonproteolytic actions of thrombin peptides.

The overlap in stimulation of early events between proteolytic activation of PAR₁ and nonproteolytic activation of a separate receptor system could provide a mechanism to maximize thrombin's initiation of tissue repair. This would also insure that if there was a signaling defect in one of the pathways, the alternative pathway would initiate the early events required for normal tissue repair. Interestingly, Connolly et al. (1997) showed that knocking out PAR₁ produced mice that had normal dermal wound healing. This finding suggests that required inflammatory responses generated through PAR₁ activation can also be activated by signaling through another PAR receptor or through nonproteolytic activation of a separate receptor.

Recent studies with PAR₁-activating peptide (PAR₁-AP) incorporated into polylactate glycolate microspheres showed an early effect on cellularity and promotion of wound closure (Rusanova et al. 2006; Stashevskaya et al. 2007). These studies suggested that the PAR₁-AP was accelerating early inflammatory and proliferative events. This is consistent with increasing evidence that thrombin activation of PAR₁, in fact, is linked to inflammation and increased production of inflammatory mediators such as TNF α (see chapter by Chen et al. in this volume). Early inflammatory events are necessary for wound repair, but excessive or prolonged inflammation delays wound healing, increases scar formation, and plays a role in formation of chronic wounds (Martin and Leibovich 2005; Eming et al. 2007). In this respect, it may be important that nonproteolytic thrombin fragments or inactive thrombin molecules generated in the postclotting wound environment can both promote and attenuate the inflammatory response.

Direct application of thrombin or TP508 significantly accelerated healing of dermal incisional wounds in rats resulting in dermal breaking strength at day 7 that was 55 and 82% greater than controls, respectively (Carney et al. 1992). In these studies, histological analysis showed that a single topical application of thrombin promoted early recruitment of inflammatory cells and stimulated revascularization more than seen in control animals. The effect of thrombin, however, was less than that achieved with a single topical application of TP508. Angiograms at day 7 showed that TP508-treated incisions had more functional blood vessels crossing the incision line than thrombin-treated or control incisions (14.5 for TP508 vs. 12.5 for thrombin and 8.7 for controls). Blinded histological scoring of these incisions at day 7 postwounding (using values of 0–5) showed that relative to control and thrombin-treated incisions, TP508-treated incisions had fewer inflammatory cells (0.30 vs. 0.83 for thrombin and 1.25 for control), and more Type 1 collagen (2.50 vs. 2.25 for thrombin and 1.10 for control) (Carney et al. 1992). Thus, nonproteolytic action of TP508 appears to have a greater effect on angiogenesis, on attenuation of inflammation, and on collagen deposition than that seen with addition of proteolytically active thrombin.

To better define the mechanisms by which TP508 stimulated wound healing, incisional wounds were made in rats that had been exposed to surface or whole body radiation (Cromack et al. 1992). In this model, TGF β accelerates healing when whole body irradiation destroys circulating inflammatory cells, while PDGF does not. In contrast, surface irradiation that blocks activation of cells residing in the skin prevents TGF β stimulation, but not that of PDGF. Interestingly, TP508 and a shorter version of this peptide, stimulated healing under both conditions, suggesting that it positively affected wound healing by both recruiting inflammatory, progenitor cells from the circulation and by activating cells resident at the site of injury (Cromack et al. 1992).

In larger full-thickness excisional dermal wounds, TP508 also accelerated healing with a single topical application of 0.1 μ g per wound (Stiernberg et al. 2000). In these studies, as in incisional wounds described earlier, TP508 treatment resulted in early recruitment of inflammatory cells and early revascularization, but resolved the inflammatory phase earlier than in controls. To determine if the same effect could be seen in wounds that more nearly reflected a chronic wound, surgical flaps were cut and lifted from the underlying muscle fascia on the backs of rats to create tissue ischemia. Wounds in these ischemic flaps heal more slowly than in normal skin, but TP508 treatment restored healing to the level seen in normal nonischemic skin (Norfleet et al. 2000b). In these wounds, as well, TP508 treatment caused early accumulation of inflammatory cells, early angiogenic responses, and an earlier resolution of the inflammatory phase of wound healing. Similar results were also seen with TP508 application to wounds in leptin receptor negative (Db/Db) mice (Warren et al. 1992), suggesting that TP508 also reverses conditions that delay wound healing in these mice.

If thrombin breakdown and release of proteolytically inactive thrombin peptides is a universal part of tissue repair, it was hypothesized that the thrombin peptide TP508 should also stimulate angiogenesis and healing in other types of tissue.

Indeed, a single injection of TP508 (1 μ g) into closed femoral fractures in rats significantly increased callus size and mechanical strength of these bones as early as 3 weeks post fracture (Wang et al. 2005). In these studies, TP508 treatment also significantly increased both vessel density and functional development of the vessels. Moreover, gene array analysis of these fracture calluses showed that TP508 induced expression of growth factors, inflammatory mediators, and angiogenesis-related genes (Wang et al. 2005). These findings were confirmed in a model of distraction osteogenesis where TP508 significantly accelerated healing with histology showing increased blood vessel development and less inflammation in TP508-treated samples relative to controls (Li et al. 2005b). Thus, in bone, as in skin, it appears that thrombin peptides initiate a cascade of molecular events that accelerate tissue repair by recruiting inflammatory cells, shortening the inflammatory stage of healing, and stimulating angiogenesis.

Additional studies showed that TP508 promoted bone formation and later stage remodeling in *critical-sized* defects where 1.5 cm of bone was cut from rabbit ulna (Sheller et al. 2004). In this *critical-sized* defect model, bone does not spontaneously form in the gap unless a three-dimensional osteoconductive matrix and an osteoinductive agent such as bone morphogenic protein are added. Thus, this is a very different type of tissue repair model from those in skin or bone fracture where tissues normally heal. In two different studies, TP508 stimulated formation of bone that filled all or most of the segmental gap (Sheller et al. 2004). In one of these studies, TP508 was delivered in microspheres for controlled release, but the volume of microspheres used was less than 5% of the gap volume, which had been created in the bone. Thus, TP508 recruited cells and established a matrix that provided an osteoconductive as well as an osteoinductive environment (Sheller et al. 2004). Surprisingly, the new bone formed in these TP508-treated defects showed signs of developing a smooth outer surface and open bone marrow space, demonstrating not only that bone had formed, but also that it was progressing toward late stages of normal bone remodeling. Thus, in bone, TP508 initiates a process that promotes late stages of tissue repair and remodeling as well as stimulating the early recruitment and proliferation of bone progenitor cells.

7.6 Clinical Studies

Studies with cultured cells and animal models demonstrated that TP508 stimulated early cellular events, angiogenesis, and later stages of healing. The peptide appeared to be quite effective in dermal wounds including those in ischemic tissues and in diabetic mice. In addition, studies in fracture repair and large critical-sized bone defects indicated that this peptide stimulated later stages of healing as well. A natural extension of these studies, and perhaps a more relevant test of the hypothesis that thrombin peptides play a key role in tissue repair, is to evaluate the effects of TP508 on wounds that have delayed healing in human clinical trials. Clinical trials were therefore initiated in subjects with diabetic foot ulcers and distal radius fractures.

7.6.1 Diabetic Foot Ulcers

A pilot, randomized, double-blind, placebo-controlled clinical trial to determine safety and potential benefit of TP508 was recently completed in subjects with diabetic foot ulcers (Fife et al. 2007). TP508 was topically applied in saline twice weekly in combination with standard-of-care treatment and offloading for up to 20 weeks. In this pilot trial, complete healing of foot ulcers was achieved in 4 of 13 of the saline placebo controls (31%) compared with 9 of 12 (75%) and 7 of 10 (70%) in the 1 and 10- μ g TP508-treatment groups, respectively. Median time to closure in the placebo group was not reached by 140 days, but was reached in 94 days in the 1- μ g group and 72 days in the 10- μ g group ($p = 0.033$). Thus, treatment with 10- μ g of TP508 twice weekly almost doubled the rate of foot ulcer closure. Moreover, this data shows that treatment with TP508 nearly doubled the probability of complete ulcer closure within 60 days. Interestingly, in a subset analysis of the most critical and most difficult to heal ulcers (those located on the heel of the foot), TP508 treatment resulted in closure of 7 out of 8 ulcers (87%), while placebo treatment resulted in no closures, or 0 out of 6 ulcers (0%). This significant effect suggests that the cellular effects of proteolytically inactive thrombin-derived peptides may stimulate a cascade of events that are highly active in reversing defects associated with some of the most severe chronic wounds.

There are a number of theories for why diabetic foot ulcers do not heal. These ulcers seem to be caught in an inflammatory phase of healing, with elevated proinflammatory cytokines, elevated metalloproteinases (MMPs), and no apparent progression of angiogenesis or epithelialization (Trengeve et al. 1999; Mi et al. 2007). In animal models, TP508 treatment produced early attenuation of the inflammatory process and promoted angiogenesis. In addition, in fibroblast cultures, TP508 inhibited the upregulation of collagenase stimulated by thrombin or TNF α . Thus, a potential explanation for the apparent effectiveness of TP508 in diabetic foot ulcer treatment may relate to its ability to attenuate the inflammatory response and reduce production of metalloproteinases.

Recent studies have indicated that failure of diabetic foot ulcers to heal also involves defective nitric oxide (NO) signaling that is required for angiogenesis, endothelial function, VEGF production, and recruitment of progenitor cells to the site of injury (Veves et al. 1998; Boykin 2000; Arana et al. 2004; Brem and Tomic-Canic 2007; Gallagher et al. 2007). Interestingly, TNF α and other proinflammatory cytokines cause loss of NO signaling by increasing levels of arginase and decreasing levels of endothelial nitric oxide synthase (Anderson et al. 2004; Gao et al. 2007). Results from our laboratory recently have shown that TP508 reverses effects of TNF α and hypoxia in human coronary artery endothelial cells to stimulate NO production and restore NO signaling (Olszewska-Pazdrak et al. 2007a,b; Fossum et al. 2008). Thus, this may be another mechanism by which thrombin peptides can reverse the effects of inflammation and promote chronic wound healing.

7.6.2 *Distal Radius Fracture Repair*

Two clinical trials have now been completed to determine the effect of TP508 on repair of distal radius fractures. TP508 was tested in a phase 1/2 prospective, double blind, randomized, placebo-controlled distal radius fracture study. In this study, a single injection of TP508 or saline vehicle alone was injected into the fracture site. Radiographic evaluation showed that the median time to healing was 23.5 days in subjects treated with 10- μ g TP508 vs. 31 days in subjects treated with placebo (Ryaby et al. 2006). Thus, fractures healed on average 25–30% faster when treated with TP508. Clinically, the time required for 50% of the fractures to heal was 10.6 days faster for subjects treated with TP508 than for those treated with placebo, and the time required for 70% to heal occurred 13.1 days faster (Ryaby et al. 2006). The second trial demonstrated significant improvement in cortical bridging and decreased healing time in subjects with extra-articular fractures, but no overall reduction in time to fixation removal (presented at the 2007 meeting of the American Academy of Orthopaedic Surgeons Meeting 2007; Ladd et al. 2007). Interestingly, this study also evaluated at-risk-populations such as those with osteopenia who demonstrated delayed fracture healing. In this population, significant effects of TP508 were seen in time to immobilization removal and in time to overall radiographic fracture healing. Thus, TP508 was especially effective in treatment of osteopenic women. These trial results support the concept that thrombin peptides such as TP508 may initiate a universal set of signals that promote healing especially when healing is impaired as it is in diabetic foot ulcers or women with osteopenia.

7.7 Conclusions

Studies with thrombin, thrombin peptides, and PAR-AP indicate that early events in wound healing involving recruitment of inflammatory cells, endothelial cell permeability changes, and angiogenesis can be stimulated by activation of PAR receptors or by nonproteolytic action of thrombin peptides. Attenuation of inflammation and promotion of later stages of healing, however, appear to be primarily stimulated by thrombin peptides.

Our working model for the involvement of two types of thrombin receptor interactions in tissue repair is presented in Fig. 7.3. This two-receptor model provides an explanation for some of the observed temporal and functional differences seen with proteolytic and nonproteolytic activation of wound healing events. For example, proteolytically active thrombin can initiate endothelial changes and inflammatory cell recruitment immediately after vascular injury. Proteolytically inactive peptide fragments can also recruit inflammatory cells, but do not have the same inflammatory activity as that seen with PAR activation. Temporally, thrombin peptides released from the clot may provide a longer term gradient to insure recruitment of inflammatory cells to the center of large wounds. Since thrombin is

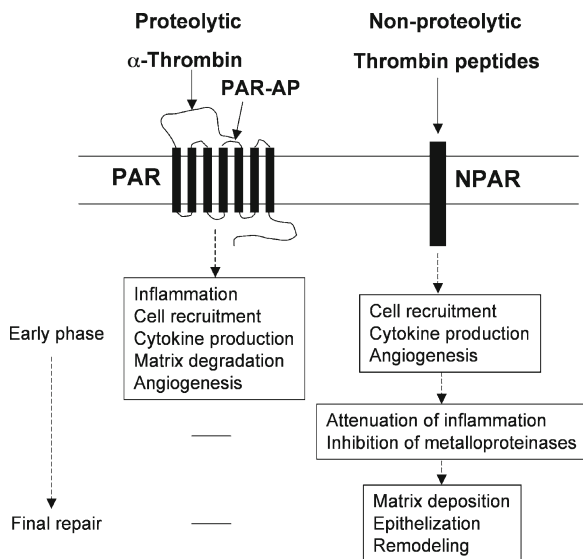


Fig. 7.3 Working model of thrombin effects on early and later stages of wound healing mediated through PAR and NPAR receptors

rapidly inactivated, attenuation of inflammation and stimulation of later healing events are much easier to explain through a separate nonproteolytic activation mechanism. Much is yet to be learned, however, about the complex involvement of thrombin in wound healing and tissue repair.

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Chapter 8

The Role of Thrombin and Thrombin Receptors in the Brain

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Abstract The serine protease thrombin is generated from its zymogen prothrombin. Both thrombin and prothrombin have been detected locally in the brain. Emerging evidence demonstrates that thrombin exerts physiological and pathological functions in the central nervous system. During brain development, thrombin regulates cell proliferation, differentiation, and migration. Thrombin is also involved in synaptic organization and synaptic plasticity in normal brain. In the brain injured in neurodegenerative disorders, the activity of thrombin is modulated and thrombin mediates the dual opposite effects. Low concentrations of thrombin rescue neural cells from death after brain insults. In contrast, thrombin at high concentrations exacerbates brain damage. The cellular functions of thrombin are mainly regulated by G protein-coupled protease-activated receptors (PARs). Thrombin can signal to PAR₁, PAR₃, and PAR₄. PAR₁ has been shown to mediate extensively thrombin-induced neurodegeneration and neuroprotection in the brain. Therefore, thrombin and thrombin receptors represent novel therapeutic targets for treating neurodegenerative diseases.

8.1 Introduction

Thrombin is generated by the cleavage of prothrombin in the presence of activated factors Xa and Va, calcium and membrane phospholipids (Grand et al. 1996). Prothrombin is mainly produced in the liver, and secreted into the bloodstream (Fenton 1986), where it is converted into mature thrombin during the coagulation cascade (Davie et al. 1991). Dihanich et al. (1991) observed that prothrombin is expressed in the brain, although the amount of neural prothrombin

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is about 1% or less compared to that in liver. In rat brain, prothrombin mRNA can be detected early in development (E13), it then decreases, but increases after birth until adulthood. After birth, prothrombin in rat brain is distributed in the olfactory bulb, cortex, colliculus superior and inferior, corpus striatum, thalamus, and hippocampus. Later on, the expression of factor X, the principle prothrombin activator, is also observed in the brain (Shikamoto and Morita 1999). In addition, thrombin inhibitors including protease nexin-1 (PN-1), antithrombin III, and plasminogen activator inhibitor type 1 are locally expressed in the brain (Deschepper et al. 1991; Kalaria et al. 1993; Hua et al. 2002). Therefore, the neural prothrombin might be the physiological source of thrombin in the brain. Indeed, the presence of thrombin in the brain has been demonstrated by several studies. Thrombin is expressed in neurons and glial cells (Deschepper et al. 1991; Arai et al. 2006).

Thrombin exerts its biological functions through both soluble target proteins and its G protein-coupled receptors, protease-activated receptors (PARs). The PAR family consists of four members (PAR_{1, 2, 3, 4}) (Vu et al. 1991; Nystedt et al. 1994; Ishihara et al. 1997; Xu et al. 1998). Thrombin can signal via PAR₁, PAR₃, and PAR₄, but not PAR₂ (Coughlin 2000). All thrombin receptors are widely expressed in various cells in the brain, including neurons, microglia, astrocytes, and oligodendrocytes (Ubl et al. 1998; Striggow et al. 2001; Suo et al. 2002a; Wang et al. 2002a, 2004; Arai et al. 2006). It has been shown by *in situ* hybridization that PAR₁ mRNA is widespread but with low intensity in the late embryonic and early postnatal nervous system, and becomes more pronounced in adult animals (Niclou et al. 1998). Immunohistochemistry reveals that PAR₃, PAR₄, and to a lesser extent PAR₁ are widely distributed in the adult rat brain tissue (Striggow et al. 2001). PAR₁ is abundant in the hippocampus, particularly in the pyramidal cell layers of the CA2 and CA3 region, and low-level expression is observed in the cortex, thalamus, hypothalamus, striatum, and amygdala. The abundant expression of PAR₃ is observed in all cortical layers, hippocampus, the medial habenular nucleus, the central amygdala, ventral thalamus, hypothalamus, and striatum. Similarly, the expression pattern of PAR₄ in the rat brain is dominant in the hippocampus, all cortical layers, thalamus, hypothalamus, and amygdala.

The local expression of thrombin and thrombin receptors in the brain indicates that thrombin and thrombin receptors exert physiological functions in the nervous system, such as neural development and plasticity. Accumulating evidence demonstrates that the level of thrombin is increased in the brain of patients with neurodegenerative diseases including stroke, Alzheimer's disease, Parkinson's disease, and human immunodeficiency virus (HIV)-associated dementia, accompanied with altered expression of PARs. Elevated thrombin inhibitor levels are observed in the brain under pathological conditions as well. The modulation of thrombin activity by its endogenous inhibitors plays an important role in the brain injury (Fig. 8.1) and, thus, thrombin and thrombin receptors have been valuable therapeutic targets for treating neurodegenerative disorders.

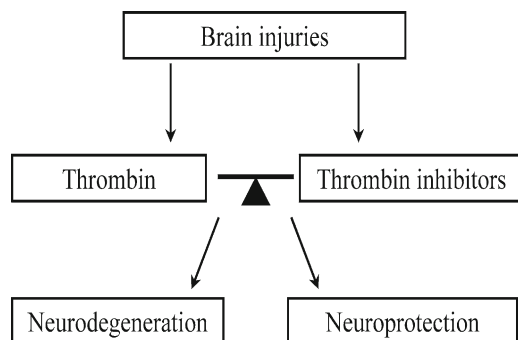


Fig. 8.1 Thrombin and thrombin inhibitors after brain injuries. Thrombin activity is significantly increased in the brain after brain injuries (e.g., ischemia, trauma, Alzheimer). High concentrations of thrombin induce neurodegeneration, whereas thrombin at the low concentration contributes to neuroprotection. On the other hand, brain injuries could also upregulate thrombin inhibitors in the brain. Thrombin inhibitors strictly regulate the effect of thrombin in the brain

8.2 Thrombin in Neural Development and Plasticity

The expression of prothrombin and PAR₁ in the brain throughout development indicates that thrombin may play an important role in neural development. Thrombin has been shown to have a direct effect on the cell morphology of astrocytes, fetal neurons, and neuroblastoma cells (Cunningham and Gurwitz 1989; Grand et al. 1989; Grabham et al. 1991; Grabham and Cunningham 1995). Low concentrations of thrombin induce neurite retraction in neurons (Zurn et al. 1988; Grand et al. 1989; Debeir et al. 1998; Olianias et al. 2007). PN-1 can reverse the effect of thrombin (Zurn et al. 1988). Thrombin also reverses the stellation in astrocytes (Suidan et al. 1997; Mahajan et al. 2000; Pai et al. 2001). The small GTP-binding protein, RhoA, has been implicated as a central molecule regulating thrombin-induced cell morphological changes in neurons and astrocytes (Fig.8.2) (Jalink et al. 1994; Mahajan et al. 2000; Pai et al. 2001). The RhoA inhibitor, C3 exoenzyme, inhibits cellular shape changes caused by thrombin. Activated RhoA translocates to the membrane, interacts with RhoA kinase, and mediates myosin light chain phosphorylation, which is required for cytoskeletal reorganization. It was shown that $\alpha_{12/13}$ and the two RhoA-specific guanine nucleotide exchange factors, oncogenic lbc and p115, mediate PAR₁ signaling to a RhoA-dependent cytoskeletal response (Majumdar et al. 1999). The PAR₁-interacting proteins, creatine kinase and heat shock protein (Hsp) 90, are required for PAR₁-induced RhoA activation (Fig. 8.2) (Mahajan et al. 2000; Pai et al. 2001). Inhibition of either creatine kinase or Hsp90 by their antisense oligonucleotides and pharmacological inhibitors abolishes thrombin-mediated cell morphological changes in astrocytes and neurons. Creatine kinase is an ATP-generating enzyme, and it might provide high-energy phosphates during cytoskeletal reorganization (Mahajan et al. 2000); whereas Hsp90, a molecular chaperone, might facilitate $\alpha_{12/13}$ coupling to the PAR₁ (Pai et al. 2001).

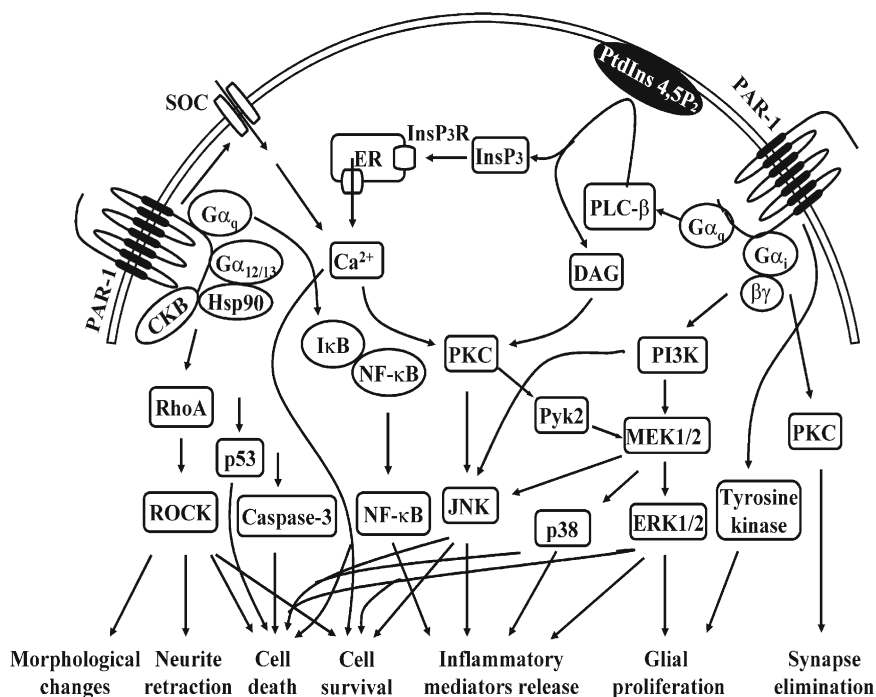


Fig. 8.2 PAR₁-mediated signal transductions in the brain. *CKB* creatine kinase, *DAG* diacylglycerol, *ER* endoplasmic reticulum, *Erk1/2* extracellular signal-regulated protein kinase 1/2, *Hsp90* heat shock protein 90, *InsP3* inositol 1,4,5-trisphosphate, *InsP3R* InsP3 receptor, *JNK* c-Jun N-terminal kinase, *MEK1/2* mitogen-activated protein kinase 1/2, *NF-κB* nuclear factor-κB, *PI3K* phosphoinositide 3-kinase, *PtdIns4,5P2* phosphatidylinositol-4,5-bisphosphate, *PKC* protein kinase C, *PLC-β* phospholipase C-β, *Pyk2* proline-rich tyrosine kinase, *ROCK* Rho kinase, *SOC* store-operated channel

Cell differentiation and proliferation are very important processes during brain development. Thrombin is considered to be a potent mitogenic agent and thereby regulates neuronal function. The proliferative effect of thrombin has been demonstrated in glial cells including astrocytes and microglia (Suo et al. 2002a; Wang et al. 2002a; Laskowski et al. 2007). Thrombin induces cell proliferation in astrocytes *in vitro* and *in vivo*, via activation of PAR₁ (Grabham and Cunningham 1995; Debeir et al. 1996; Wang et al. 2002a; Nicole et al. 2005). The involvement of PAR₃ and PAR₄ in astrocytic proliferation has been ruled out, although both receptors can be activated by thrombin (Wang et al. 2002a). Grabham and Cunningham (1995) initially found that thrombin-evoked astrocytic proliferation is dependent upon PAR₁-activated tyrosine kinase activity (Fig. 8.2). Debeir et al. (1996) demonstrated that Gα_{i/o} and protein kinase C (PKC) are involved in thrombin-induced cell division in astrocytes (Fig. 8.2). However, studies in 1321N1 astrocytoma cells suggest that Gα_{i/o} does not account for thrombin-stimulated gene expression and DNA synthesis. Instead, PAR₁ preferentially couples to Gα₁₂ to initiate transcription factor activator protein

1-mediated mitogenesis by utilizing Shc to propagate signal transduction (Collins et al. 1997). Further studies elucidated the detailed signaling pathway underlying PAR₁-mediated proliferation in astrocytes (Wang et al. 2002b; Wang and Reiser 2003). Thrombin, via activation of PAR₁, rapidly results in phosphorylation of extracellular signal-regulated protein kinase 1/2 (Erk1/2) in astrocytes (Wang et al. 2002b). The mitogen-activated protein kinase (MAPK) kinase inhibitor PD-98059 completely blocks PAR₁-mediated proliferation in astrocytes. In addition, it was found that a G $\alpha_{i/o}$ -mediated phosphoinositide 3-kinase pathway and a G α_q -mediated phospholipase C/Ca²⁺/PKC pathway are involved as upstream factors of Erk1/2 (Fig. 8.2). Interestingly, proline-rich tyrosine kinase, a nonreceptor tyrosine kinase that is implied to connect G protein-coupled receptors to Erk1/2 activation, is also phosphorylated in response to thrombin. Proline-rich tyrosine kinase links a PAR₁-dependent increase in cytosolic calcium and PKC activation to the Src tyrosine kinase-mediated ERK pathway by recruiting the adapter protein Grb2 (Fig. 8.2) (Wang and Reiser 2003). This signaling cascade, however, does not depend upon transactivation of epidermal growth factor receptor (Wang et al. 2002b), although it was shown that transactivation of epidermal growth factor receptor by thrombin is important for MAPK activation in Rat-1 cells and smooth muscle cells (Daub et al. 1996; Kalmes et al. 2000).

Studies using an in vitro model of the mammalian neuromuscular junction reveal that thrombin regulates synaptogenesis in the peripheral nervous system. Inhibition of thrombin by hirudin and PN-1 blocks synapse elimination (Liu et al. 1994). PAR₁-induced pertussis toxin-sensitive G protein and PKC might be involved in thrombin-mediated synapse loss (Fig. 8.2) (Lanuza et al. 2001, 2003). Synapse remodeling is a critical process during development of the nervous system. These data suggest that thrombin is important in synaptic organization during development.

Serine proteases have been suggested to regulate synaptic plasticity and learning and memory in the brain (Tomimatsu et al. 2002). Overexpression of tissue plasminogen activator (tPA) in neurons could enhance long-term potentiation and thereby improve learning and memory-dependent processes (Madani et al. 1999). Recent studies indicate the role of thrombin in learning and memory. Activation of PAR₁ induces glutamate release from astrocytes (Lee et al. 2007). The release of glutamate is dependent on PAR₁-mediated intracellular calcium rise. Importantly, the elevated extracellular glutamate activates *N*-methyl-d-aspartate (NMDA) receptors on neighboring neurons in hippocampal slices (Lee et al. 2007). Therefore, thrombin can potentiate NMDA receptor function in hippocampal neurons (Gingrich et al. 2000). The glutamate receptor plays a crucial role in synaptic plasticity (Derkach et al. 2007). A recent study by neurological behavior analysis demonstrates that PAR₁ deficiency in mice impairs learning, passive avoidance behavior, and fear-conditioned freezing, but not other neurological types of behavior (Almonte et al. 2007). Therefore, thrombin-induced glutamate release from astrocytes might regulate learning and memory in normal brain. However, high concentrations of thrombin are neurotoxic and cause impairment of reference memory. Thrombin-induced memory impairment is not due to impaired motor function (Mhatre et al. 2004).

8.3 Thrombin in Neuroinflammation

Over the past years, thrombin is increasingly considered as a proinflammatory agent, because it is able to stimulate inflammatory cells to release pro-inflammatory mediators. In the brain, microglia cells are the major immune effector cells and activated microglia participate in neuroinflammation around the injured area in the brain. It has been shown that thrombin mediates the expression of inducible nitric oxide synthase (iNOS) and several pro-inflammatory cytokines including interleukin (IL)-6, IL-1 α , IL-1 β , and tumor necrosis factor (TNF)- α , and induces microglial activation *in vitro* and *in vivo* (Suo et al. 2002b; Carreno-Muller et al. 2003; Choi et al. 2003a, 2005; Katsuki et al. 2006; Fujimoto et al. 2007). PKC is involved in thrombin-induced nitric oxide (NO) release from microglia. Inhibition of Erk1/2 and p38 MAPK significantly reduces NO production caused by thrombin in microglia (Ryu et al. 2000). In addition, nuclear factor- κ B (NF- κ B) is rapidly activated upon thrombin stimulation and is required for iNOS expression and subsequent NO release in microglia (Fig. 8.2) (Ryu et al. 2000). On the other hand, in microglia, thrombin also induces expression of suppressor of cytokine signaling 3 (SOCS3), which is known as negative feedback regulator of inflammation (Yang et al. 2004). PKC δ regulates thrombin-induced SOCS3 expression. Similarly, thrombin triggers the immunosuppressive cytokine IL-10 release from rat microglia, which in turn inhibits TNF- α release (Kim et al. 2002). Therefore, the expression of SOCS3 and IL-10 might prevent thrombin-mediated prolonged inflammatory responses in microglia. Nevertheless, thrombin-induced expression of iNOS and cyclooxygenase-2 (COX-2) in microglia causes cell death of dopaminergic neurons in the substantia nigra of rat brain (Choi et al. 2003a). Thrombin-induced neuronal death is partially inhibited by either NOS inhibitor L-NAME or COX-2 inhibitor DuP-697 *in vivo*. Importantly, Erk1/2 and p38 MAPK regulate expression of iNOS and COX-2, and inhibition of both MAPKs also rescues dopaminergic neurons in the substantia nigra caused by thrombin (Choi et al. 2003a). Likewise, thrombin activates NADPH oxidase, an important source of reactive oxygen species (ROS) during inflammation, in microglia in the hippocampus (Choi et al. 2005). NADPH oxidase contributes to thrombin-induced hippocampal neuronal death *in vivo*. Therefore, neurodegenerative disorders are significantly associated with thrombin-induced neuroinflammation in microglia, such as in Parkinson's disease and Alzheimer's disease. However, the involvement of PARs in thrombin-induced microglial activation is still complex. Suo et al. (2002a) demonstrated that PAR₁ partially contributes to microglial proliferation, but is not involved in thrombin-induced TNF- α release. Instead, PAR₄ induces prolonged rise in intracellular calcium concentration, Erk1/2 activation, and NF- κ B activation, thereby mediating thrombin-induced TNF- α release and microglial activation (Suo et al. 2002b). Conversely, many studies demonstrate that specific PAR-activating peptide agonists cannot induce NO release and expression of proinflammatory cytokines in cultured microglia, and that microglial activation by thrombin is independent of PARs (Ryu et al. 2000; Lee et al. 2005, 2006). It was also shown that the specific thrombin inhibitor hirudin has no effect on thrombin-induced

neuronal death and NO production in co-cultures of cortical microglia and neurons, suggesting that a nonproteolytic activity of thrombin contributes to microglial activation (Lee et al. 2006). Hanisch et al. (2004) further revealed that a high molecular weight contaminant of thrombin preparations, but not thrombin itself, induces cytokine release and proliferation in microglia. Therefore, the effect of thrombin on microglia is very controversial. Future studies on transgenic mice may reveal the precise thrombin effects on microglia.

Astrocytes, the major glial cell type in the brain, provide tropic factors and energy for neurons, and maintain extracellular ions and neurotransmitters. Now astrocytes are also thought to be important neural cell types that are involved in inflammatory reactions in the brain. Thrombin, acting on PAR, induces the release of arachidonic acid, an important proinflammatory mediator, in human astrocytoma cells (Hernandez et al. 1997) and in rat astrocytes (Strokin et al. 2003). In addition, thrombin- or PAR₁ AP-activated PAR₁ also significantly increases mRNA expression of IL-6, IL-1 β , and TNF- α (Boven et al. 2003; Fan et al. 2005) and elevates NO produced by iNOS in astrocytoma cells (Meli et al. 2001; Boven et al. 2003). In vivo studies have shown that iNOS can be detected in astrocytes and microglia in mouse striatum injected with PAR₁ AP (Boven et al. 2003). Therefore, activation of astrocytes by thrombin also contributes to neuroinflammation in the brain.

Recently, Wang et al. demonstrated that, in rat astrocytes, thrombin concentration dependently induces the secretion of the chemokine growth-regulated oncogene/cytokine-induced neutrophil chemoattractant-1 (GRO/CINC-1), a counterpart of the human GRO and IL-8 (Wang et al. 2006a, 2007c). PAR₁ predominantly mediates thrombin-induced GRO/CINC-1 release from astrocytes. Importantly, c-Jun N-terminal kinase (JNK) isoforms 2 and 3, but not JNK1, regulate this thrombin effect (Wang et al. 2007c). In addition, Erk1/2 is also partially involved. Furthermore, it was found that PAR₁-induced PKC activation is required for JNK2 phosphorylation, whereas phosphoinositide 3-kinase is the upstream kinase of JNK3 (Fig. 8.2) (Wang et al. 2006a, 2007c). In addition to activation of MAPKs, thrombin also evokes intracellular calcium rise in multiple cell types, by regulating the PAR₁-mediated phospholipase C- β pathway and calcium channels (Fig. 8.2) (Wang et al. 2002a, 2004; Noorbakhsh et al. 2003; Luo et al. 2005). Interestingly, calcium exerts a dual role in PAR₁-induced GRO/CINC-1 secretion in astrocytes (Wang et al. 2007a). An increase of cytosolic calcium negatively regulates PAR₁-induced GRO/CINC-1 gene expression in rat astrocytes, but on the other side the basal level of calcium is the basic prerequisite for GRO/CINC-1 protein synthesis and secretion.

Significantly, the released GRO/CINC-1 caused by thrombin enhances cell survival in astrocytes (Wang et al. 2006a). GRO/CINC-1 inhibits ceramide-induced cytochrome c release from mitochondria, and thereby prevents apoptosis in astrocytes. The GRO/CINC-1 receptor antagonist completely abolishes the protective effect of thrombin in astrocytes. Interestingly, CINC-3, another member of the CINC family, is also significantly released from astrocytes upon activation of PAR₁ or PAR₄. This chemokine exerts the same protective effects on cell survival, as CINC-1 in astrocytes (Wang et al. 2007b). Recent studies revealed that the protective mechanisms can be extended to neurons. It was found that CINC

released from astrocytes upon thrombin stimulation protect cortical neurons from ceramide-induced apoptosis (Wang et al. 2007b). Therefore, CINC_s are likely to be responsible for the protective action of thrombin after brain injury. The proinflammatory factor GRO/CINC-1 is expressed by inflammatory cells at the site of inflammation and could be transiently increased in ischaemic brain areas, resulting in granulocyte infiltration into the brain in response to focal ischaemia, as shown in rats (Yamasaki et al. 1995). The level of thrombin is also increased after brain injuries, including ischemia (Riek-Burchardt et al. 2002). Thus, the increased CINC_s might result from PAR_s activation by thrombin, and they can prevent apoptosis of neurons and astrocytes due to brain insults.

8.4 Thrombin in Neurodegenerative Disorders

8.4.1 *Thrombin and Stroke*

The serine protease tPA has been developed as an important therapeutic tool in the clinic. Recombinant tPA has been used to treat ischemic stroke patients, because it is involved in degrading fibrin clots and thereby improves patients' outcome after ischemic stroke (Qureshi 1996). Like tPA, thrombin has been demonstrated to modulate ischemic, hemorrhagic, and traumatic brain injury. High levels of thrombin have been detected in the ischemic brain (Riek-Burchardt et al. 2002; Xi et al. 2002). In addition, blood–brain barrier disruption, which is often associated with ischemic and traumatic brain injury, enables the bloodstream thrombin to infuse into the brain parenchyma. On the other side, experimental ischemia conditions have been identified to regulate differentially the expression of thrombin receptors in the brain. It was shown that the expression of PAR₁ and PAR₃ is increased in hippocampal slices after exposure to oxygen–glucose deprivation (OGD) (Strigrow et al. 2001). Transient focal ischemia induced by microinjection of endothelin-1 near the middle cerebral artery results in PAR₁ downregulation (Rohatgi et al. 2004). However, the PAR₄ mRNA level is increased 12 h after ischemia. Unlike the other PAR_s, PAR₃ is upregulated transiently and then downregulated after transient focal ischemia (Rohatgi et al. 2004). Recently, it was shown that focal ischemia induces expression of PAR₁ and PAR₃ on microglia and enhances PAR₄ labeling in the penumbra (Henrich-Noack et al. 2006a). These data strongly indicate the important role of thrombin and thrombin receptors in stroke.

Evidence accumulating over recent years demonstrates that low concentrations of thrombin are neuroprotective in the ischemic brain. In vitro studies have shown that thrombin (10 pM–10 nM) protects hippocampal neurons and astrocytes from cell death in response to OGD, hypoglycemia, growth supplement deprivation, or oxidative stress (Vaughan et al. 1995; Strigrow et al. 2000). These effects of thrombin are mediated by PAR₁ and can be inhibited by hirudin. Thrombin, via PAR₁-induced CINC_s release, prevents cell death of cortical neurons and astrocytes caused by ceramide (Wang et al. 2006a), which is increased in the brain after brain

injuries (e.g., trauma, ischemia) (Ohtani et al. 2004). The neuroprotective effect of thrombin is also supported by *in vivo* studies. It was demonstrated that intracerebral infusion of a low dose of thrombin attenuates the brain edema after intracerebral hemorrhage, a subtype of stroke (Xi et al. 2000). This phenomenon is the so-called thrombin preconditioning (TPC). Likewise, TPC also inhibits the brain edema formation caused by a large dose of thrombin, lysed erythrocytes or iron (Xi et al. 1999; Hua et al. 2003a), and reduces infarct volume after focal cerebral ischemia (Masada et al. 2000; Henrich-Noack et al. 2006b). The maximal effect of TPC on brain edema formation peaks at 7 days after pretreatment (Xi et al. 1999). The co-injection of hirudin abolishes the protective effect of TPC in animal models (Xi et al. 1999; Masada et al. 2000). Further, it was shown that the PAR₁ antagonist RPPGF has effects similar to those of hirudin (Jiang et al. 2002), suggesting that activation of PAR₁ is required for thrombin-induced brain tolerance. The neurological behavior test indicates that TPC enhances the capacity of mice on motor performance after transient focal cerebral ischemia (Granziera et al. 2007). Studies on the signaling mechanism of TPC reveal that activation of Erk1/2 is involved, since Erk1/2 is phosphorylated in the ipsilateral basal ganglia after TPC and the MAPK kinase inhibitor PD-98059 can block the protective effect of TPC (Xi et al. 2001; Jiang et al. 2002). TPC is also significantly associated with the enhanced expression of Hsp27, hypoxia inducible factor-1 α (HIF-1 α), transferrin, and transferrin receptor in the injured brain, indicating the neuroprotective effects of Hsp27, HIF-1 α , transferrin, and transferrin receptor in stroke (Xi et al. 1999; Hua et al. 2003a). Recently, it was found that ceruloplasmin, which can regulate iron metabolism by oxidizing ferrous iron to ferric iron, is increased in the ipsilateral basal ganglia after TPC. The injection of exogenous ceruloplasmin reduces ferrous iron-induced brain edema, suggesting that ceruloplasmin upregulation may be one of mechanisms for thrombin-induced brain tolerance (Yang et al. 2006). In addition, it was reported that the JNK inhibitors prevent thrombin-induced ischemic tolerance in hippocampal slices exposed to OGD. The involvement of JNK was further documented in a mouse model of transient focal cerebral ischemia (Granziera et al. 2007). These findings are supported by studies from Wang et al. showing that JNK predominantly regulates PAR₁-induced CINC-1 secretion, which is a protective chemokine in cortical neurons and astrocytes (Wang et al. 2006a, 2007c).

Although low concentrations of thrombin protect the brain from insults, thrombin at high concentrations (≥ 100 nM) causes brain damage (Xi et al. 2003). In the hippocampal slice cultures, high concentrations of thrombin exacerbate OGD-induced neuronal death. Moreover, thrombin at 500 nM alone induces more severe cellular damage than OGD alone. A sustained calcium response was observed in thrombin-treated hippocampal slices, which might be associated with thrombin-mediated neurodegeneration (Strigrow et al. 2000). Thrombin also directly induces delayed neuronal injury in the cerebral cortex and shrinkage of the striatum in organotypic cortico-striatal slice cultures (Fujimoto et al. 2006). Thrombin-induced shrinkage of the striatum is inhibited by the thrombin inhibitor argatroban and the PAR₁ antagonist FR171113, whereas thrombin-induced cortical injury is only partially attenuated by argatroban (Fujimoto et al. 2006). This suggests

that PAR₁ differently mediates the toxic effect of thrombin in the cortex and striatum. Thrombin-induced striatal neuronal death is further confirmed *in vivo* (Fujimoto et al. 2007). The activated microglia is involved in thrombin neurotoxicity. These results are consistent with similar previous findings (Xue and Del Bigio 2001).

It was also shown that thrombin is responsible for brain edema formation after intracerebral hemorrhage (Lee et al. 1996) or after focal cerebral ischemia (Hua et al. 2003b). Inhibition of thrombin by argatroban reduces neuronal death and brain edema, and improves survival ratio and stroke index in animal models (Ohyama et al. 2001; Kitaoka et al. 2002; Ohnishi et al. 2007). Recently, it was found that the level of TNF- α is increased in the brain after intracerebral hemorrhage. The increasing TNF- α contributes to acute brain injury, since intracerebral hemorrhage-induced brain edema and neurological deficits are less pronounced in TNF- α knockout mice (Hua et al. 2006). Interestingly, intracerebral infusion of thrombin also results in TNF- α upregulation. However, the effect of thrombin-induced TNF- α in the injured brain is still unknown (Hua et al. 2006). A variety of proinflammatory mediators are generated in the brain under ischemic conditions, and contribute to brain damage as well as mediate neuroprotection (Trendelenburg and Dirnagl 2005). Overexpression of the IL-1 receptor antagonist in the brain reduces brain edema after intracerebral hemorrhage (Masada et al. 2003). Thrombin is able to induce the release of proinflammatory factors in various cell types (Suo et al. 2002a; Choi et al. 2003a; Coughlin and Camerer 2003). Therefore, thrombin-induced neuroinflammation may be involved in neurodegeneration in stroke.

Iron is a major factor involved in brain damage after intracerebral hemorrhage (Hua et al. 2007). Iron is increased after intracerebral hemorrhage, and intracerebral infusion of iron causes brain edema (Huang et al. 2002). Iron-loaded transferrin can exacerbate thrombin-induced brain edema even when thrombin was applied at a low concentration of 5 nM (Nakamura et al. 2005), which is shown to exert a protective effect in stroke (Xi et al. 2000). In addition, thrombin and matrix metalloproteinase-9 (MMP-9) collaborate to promote neuronal death (Xue et al. 2006). The neurotoxicity of thrombin involves activation of PAR₁ and MMP-9. MMP-9 can degrade extracellular matrix and cause blood–brain barrier disruption (Svedin et al. 2007). MMP-9 is increased in the brain after intracerebral hemorrhage, and intracerebral hemorrhage-induced brain injury is significantly reduced in MMP-9 knockout mice (Xue et al. 2006). Importantly, injection of thrombin causes more severe brain damage in wild-type mice than in MMP-9 knockout mice. Moreover, hirudin significantly reduces neurodegeneration caused by intracerebral hemorrhage in both wild-type- and MMP-9-deficient mice (Xue et al. 2006). Altogether, the neurodegenerative effect of thrombin in stroke may be enhanced by several toxic factors.

The detrimental effect of thrombin has been also demonstrated in other experimental ischemic models. *In vivo* studies demonstrate that PAR₁ activation significantly increases infarct volume in the brain after transient focal cerebral ischemia and contributes to neuronal damage, which could be prevented in PAR₁ knockout mice (Junge et al. 2003). Anatomic analysis shows no detectable difference between wild-type- and PAR₁-deficient mice in cerebrovasculature,

capillary density, or capillary diameter in the brain. Similar neuronal damage caused by PAR₁ activation was observed in mice subjected to combined hypoxia/ischemia (Olson et al. 2004). PAR₁-deficient mice have less neuronal death, reduced glial fibrillary acidic protein (GFAP) expression, and smaller lesion volumes than wild-type mice after hypoxia/ischemia. Importantly, animal behavioral studies demonstrate that PAR₁ deficiency attenuates the motor behavioral impairment in hypoxic/ischemic mice (Olson et al. 2004).

Traumatic or ischemic brain injury increases extracellular levels of the excitatory amino acid glutamate. The glutamate through its receptors exerts excitotoxicity in the brain (Kwak and Weiss 2006), besides its role in synaptic plasticity (Liu and Zukin 2007). Interestingly, it was shown that activation of PAR₁ induces glutamate release from astrocytes (Lee et al. 2007). Thrombin-induced glutamate efflux can be enhanced when astrocytes are exposed to hyposmotic media (Ramos-Mandujano et al. 2007). As a result, the NMDA receptor is potentiated by thrombin in the hippocampus, which may mediate neuronal death after blood–brain barrier breakdown under certain pathological conditions, like cerebral ischemia and trauma (Gingrich et al. 2000). Indeed, conditioned media from thrombin-stimulated astrocytes reduce neuronal survival. The neurotoxicity could be partially blocked by the NMDA receptor antagonist MK-801 (Boven et al. 2003). However, it was also shown that thrombin protects hippocampal neurons from glutamate-induced excitotoxicity (Gorbacheva et al. 2006). The PAR₁ antagonist could abolish the protective effect of thrombin.

The cellular mechanisms of thrombin toxicity reveal that apoptosis underlies the thrombin-mediated neurodegeneration. As previously shown, thrombin induces apoptosis in hippocampal neurons through activation of PAR₁ (Donovan et al. 1997). RhoA activation plays an important role in this signaling pathway (Fig. 8.2). The RhoA inhibitor exoenzyme C3 could block thrombin-induced neuronal death (Donovan et al. 1997). Recently, cell cycle proteins, cyclin D1 and cyclin-dependent kinase 4 (cdk4), were shown to be linked to thrombin-induced apoptosis in cortical neurons (Rao et al. 2007). Cyclin D1 and cdk4 have been demonstrated to mediate ischemic/hypoxic neuronal death *in vitro* and *in vivo* (Rashidian et al. 2005). Moreover, cdk4 regulates thrombin-induced Bim expression (Rao et al. 2007), a pro-apoptotic protein that is critical for neuronal apoptosis (Putchu et al. 2001). These results indicate the importance of cell cycle regulators in mediating thrombin's neurotoxic effect.

It is well known that MAPKs mediate diverse biological processes and cellular responses. Numerous studies have demonstrated that activation of PARs initiates the signaling cascade of MAPKs, including Erk1/2, JNK, and p38 MAPK, in neural cells (Suo et al. 2002a; Wang et al. 2002b, 2007c). Activation of PAR₁ leads to astrogliosis associated with the glial scar formation after brain injury *in vivo*, via the Erk1/2 pathway (Fig. 8.2; Nicole et al. 2005). Astrogliosis is considered to be a barrier to neuroregeneration and is associated with neurodegeneration after traumatic or ischemic brain injury. Therefore, astroglial Erk1/2 activation caused by PARs might result in neuronal cell loss in stroke. In addition, inhibition of Erk1/2 activation in neurons and microglia or blockade of JNK activation by their specific inhibitors reduces neuron loss in the central and peripheral regions of hematoma after

intracerebral hemorrhage, although these inhibitors have no effect on hematoma size and brain edema (Ohnishi et al. 2007).

Taken together, the signaling molecules like RhoA and MAPKs are involved in both thrombin-mediated neurodegeneration and neuroprotection in stroke (Fig. 10.2). Thus, cell death and cell survival may share initial signaling proteins, but differences in the amplitude as well as the duration of the signal may result in opposite final consequences (Donovan and Cunningham 1998).

8.4.2 Thrombin and Alzheimer's Disease

Previously, it was reported that thrombin is detected in brains of patients with Alzheimer's disease (Akiyama et al. 1992). Thrombin staining is present in senile plaques, some diffuse amyloid deposits and neurofibrillary tangles in injured brains. This finding is confirmed by recent studies, showing that prothrombin and thrombin are expressed in neural cells at both mRNA and protein levels, and they are accumulated with both extracellular and intracellular neurofibrillary tangles, senile plaques, and reactive microglial cells in the brain of Alzheimer's disease (Arai et al. 2006; Grammas et al. 2006). In addition, the expression of PARs is modulated in the rat hippocampus treated with trimethyltin that mimicks effects of Alzheimer's disease in brain tissue. Following the administration of trimethyltin, PAR₁, and to a lesser extent PAR₃ and PAR₄, are upregulated in reactive hippocampal astrocytes (Pompili et al. 2004). On the other hand, the neural thrombin inhibitor PN-1 is significantly reduced in Alzheimer's disease brain, especially in the hippocampus (Wagner et al. 1989; Vaughan et al. 1994; Choi et al. 1995). The reduction of PN-1 activity is due to the formation of the PN-1–thrombin complex in the brain, since the PN-1 mRNA level is not changed in Alzheimer's disease brain (Wagner et al. 1989; Vaughan et al. 1994). Therefore, these findings indicate that thrombin plays an important role in the pathogenesis of Alzheimer's disease.

Intracellular aggregates of the microtubule-associated protein tau are one of the pathological hallmarks of Alzheimer's disease. Thrombin is able to process tau in vitro (Arai et al. 2005). Biochemical analysis reveals that thrombin hydrolyzes tau at multiple arginine and lysine sites, including Arg¹⁵⁵-Gly¹⁵⁶, Arg²⁰⁹-Ser²¹⁰, Arg²³⁰-Thr²³¹, Lys²⁵⁷-Ser²⁵⁸, and Lys³⁴⁰-Ser³⁴¹. The thrombin-specific inhibitor PPACK completely prevents tau breakdown. Interestingly, thrombin fails to degrade phosphorylated tau induced by glycogen synthase kinase-3 β . Similarly, paired helical filament tau prepared from Alzheimer's disease brain is more resistant to thrombin proteolysis than following dephosphorylation of tau. Therefore, thrombin may be involved in the release of the N-terminal tau fragments to the cerebrospinal fluid, but induce intracellular aggregates of C-terminal tau fragments in the brain under pathological conditions. Indeed, thrombin has been shown to induce hyperphosphorylation and aggregation of tau in hippocampal neurons (Suo et al. 2003). Thrombin-induced tau aggregation is mediated by PAR₁ and PAR₄. Inhibition of Erk1/2 by PD-98059 completely abolishes thrombin-induced tau hyperphosphorylation and aggregation, suggesting that Erk1/2

is involved in this pathological process. Furthermore, it was shown that thrombin-induced tau aggregation is neurotoxic and contributes to apoptosis of hippocampal neurons (Suo et al. 2003). Taken together, thrombin might directly act on hippocampal neurons to induce neurofibrillary degeneration and contribute to the pathogenesis of Alzheimer's disease.

A β is significantly involved in initiation and progression of Alzheimer's disease. Thrombin appears to cleave amyloid precursor protein (APP) *in vitro*, thereby generating A β (Igarashi et al. 1992; Chong et al. 1994). However, several studies demonstrate that thrombin cannot induce APP processing in neurons and glioblastoma cells (Davis-Salinas et al. 1994; Brewer 1996). The effect of thrombin on A β -induced toxicity has been also investigated. It was shown that thrombin enhances A β -induced neurotoxicity via increased intracellular calcium levels and oxidative stress, whereas PN-1 protects neurons against A β toxicity (Smith-Swintosky et al. 1995). Conversely, Pike et al. (1996) reported that thrombin attenuates A β -induced cell death of hippocampal neurons. Attenuation of A β toxicity by thrombin is mimicked by PAR₁-activating peptide and can also be blocked by thrombin inhibitor hirudin and PN-1, indicating that PAR₁ mediates this thrombin effect. In addition, thrombin, through activation of PAR₁, reverses A β -induced astrocyte stellation (Pike et al. 1996). Therefore, the effect of thrombin on APP and A β is obscure and needs further investigations.

Many studies have demonstrated that microglial activation is detected in the brains of patients with Alzheimer's disease (Benveniste et al. 2001). *In vivo* studies demonstrate that thrombin induces ROS production mediated by activation of the microglial NADPH oxidase in the hippocampus (Choi et al. 2005). Furthermore, the level of protein carbonyls is significantly increased in the hippocampus 48 h after intrahippocampal injection of thrombin, suggesting that the ROS generation caused by thrombin induces oxidation of proteins. Importantly, the increased ROS level contributes to thrombin-induced loss of hippocampal neurons, which can be partially inhibited by either the antioxidant trolox or the NADPH oxidase inhibitor DPI *in vivo* (Choi et al. 2005).

In addition to oxidative stress, inflammation is also significantly associated with Alzheimer's disease (Heneka and O'Banion 2007). The inflammatory proteins such as IL-8, integrins $\alpha v \beta 3$ and $\alpha v \beta 5$ are strongly expressed in the brain of patients with Alzheimer's disease (Grammas et al. 2006). In cultured neurons, the inflammatory proteins cause ROS generation and cell death (Christov et al. 2004). Recent work further demonstrates that the expression of the transcription factor HIF-1 α , which regulates pro-inflammatory gene expression under hypoxia/ischemia, is increased in microvessels of Alzheimer's disease brain (Grammas et al. 2006). Likewise, high levels of tissue inhibitor of matrix metalloproteinases-1 are also detected in Alzheimer's disease brain (Thirumangalakudi et al. 2006). Thrombin induces the upregulation of both HIF-1 α and tissue inhibitor of matrix metalloproteinases-1 in brain endothelial cells (Grammas et al. 2006; Thirumangalakudi et al. 2006). It has been shown that endothelial cells in the injured brain are able to release thrombin (Grammas et al. 2004). Therefore, thrombin-induced alterations of the microcirculation in the brain might contribute to pathological processes of Alzheimer's disease.

However, it is not clear whether PARs are involved in microvascular abnormalities in Alzheimer's disease brain.

Emerging data strongly suggest that apolipoprotein E-4 (apoE-4) is a major risk factor for Alzheimer's disease (Roberson and Mucke 2006). ApoE-4 is localized in the senile plaques, congophilic angiopathy, and neurofibrillary tangles of Alzheimer's disease, and the interactions between apoE and A β are critical for the development of A β deposits (Strittmatter et al. 1993). Chronic exposure of high concentrations of thrombin significantly increases the expression of apoE-4 in rat hippocampus (Mhatre et al. 2006). On the other side, chronic infusion of human apoE-4 results in A β deposits surrounded by numerous microglial cells in rat brain, and increases GFAP-positive glial cells in the cortical layers and hippocampus. The cognitive functions evaluated by Morris water maze tests are also impaired in rats infused with apoE-4 (Mhatre et al. 2006). Therefore, the increased thrombin after brain injuries may contribute to the development of Alzheimer's disease through regulating apoE-4 expression.

Although in vitro studies have indicated the role of thrombin and PARs on tau aggregation and neuronal death, it is still unclear whether thrombin contributes to the formation of amyloid plaques and neurofibrillary tangles, and neurodegeneration in vivo. Further investigation in animal models may provide insights into thrombin and PARs in Alzheimer's disease.

8.4.3 *Thrombin and Parkinson's Disease*

Recent studies demonstrate that thrombin has been linked to an important neurodegenerative disease, Parkinson's disease. Parkinson's disease is characterized by the progressive and selective loss of dopaminergic neurons in the substantia nigra (Olanow and Tatton 1999). Ishida et al. (2006) found that thrombin is significantly increased in the vessel wall in the substantia nigra pars compacta of Parkinson's disease brains. The strong immunoreactivity of prothrombin is predominantly found in GFAP-positive astrocytes of Parkinson's disease brains. In addition, PAR₁ is also upregulated in GFAP-positive astrocytes due to cell proliferation in the substantia nigra pars compacta of Parkinson's disease brains (Ishida et al. 2006).

Thrombin has been shown to mediate cell death of dopaminergic neurons in vitro (Lee et al. 2005; Katsuki et al. 2006). In vivo studies further demonstrated that injection of thrombin into the substantia nigra decreased the immunoreactivity of tyrosine hydroxylase-positive dopaminergic neurons after 7 days (Carreno-Muller et al. 2003; Choi et al. 2003a). Thrombin-induced injury could be observed for up to 2 months. The application of the thrombin inhibitor α -NAPAP significantly prevents thrombin-induced loss of dopaminergic neurons in the substantia nigra (Carreno-Muller et al. 2003). Oxidative stress and neuroinflammation are the most significant pathological features of Parkinson's disease (Castano et al. 1998; Jenner 2003). Profound activation of microglia in the substantia nigra was observed after thrombin injection. The time window of microglial activation is from 4 h up to 24

h after injection, which is an early event before cell death of nigral dopaminergic neurons (Choi et al. 2003a). Importantly, activated microglia release proinflammatory factors including NO, IL-1 β , IL-6, and TNF- α in response to thrombin. Inhibition of iNOS, COX-2, or NADPH oxidase protects dopaminergic neurons. Thrombin-induced activation of Erk1/2, JNK, and p38 MAPK also contributes to degeneration of dopaminergic neurons in the substantia nigra, since their inhibitors promote cell survival against thrombin-induced injury (Choi et al. 2003a, b, 2005; Lee et al. 2005; Katsuki et al. 2006). In addition, thrombin induces caspase-3 activation and p53 upregulation in the nigral dopaminergic neurons (Choi et al. 2003b). Both death-related proteins mediate the neurodegenerative effect of thrombin. Accompanied with increased apoptotic effectors, the anti-apoptotic Bcl-2 protein is significantly reduced in the substantia nigra after thrombin injection. Although PARs are able to induce MAPK activation and release of proinflammatory factors, thrombin exerts its neurotoxic effect on dopaminergic neurons via the non-PAR mechanism (Choi et al. 2003b). Nevertheless, thrombin appears to be involved in pathological processes of Parkinson's disease.

Conversely, Cannon et al. found that PAR₁ activated by thrombin mediates neuroprotection in the rat 6-hydroxydopamine (6-OHDA) model of Parkinson's disease (Cannon et al. 2005, 2006). TPC, given at 3 days prior to 6-OHDA injection, reduces dopaminergic terminal cell loss, although it has no effect on dopamine depletion (Cannon et al. 2005). By behavioral tests, it was detected that TPC attenuates neurological deficits in the animal model of Parkinson's disease. Preconditioning with the PAR₁ peptide agonist can mimic the neuroprotective effect of thrombin, which is abolished by PAR₁ antagonists (Cannon et al. 2006). Similarly, delayed thrombin administration, at 1 or 7 days after 6-OHDA injection, also prevents 6-OHDA-induced behavioral and neurochemical deficits (Cannon et al. 2007). However, thrombin or the PAR₁ peptide agonist, co-administered with 6-OHDA, significantly increases in behavioral deficits (Cannon et al. 2007). Therefore, the neuroprotective effect of thrombin depends on the time of administration in the 6-OHDA model of Parkinson's disease.

8.4.4 Thrombin and Multiple Sclerosis

Multiple sclerosis (MS) is an inflammatory demyelinating disorder of the central nervous system. Previous studies demonstrated that the activation of the coagulation cascade, as reflected by accumulation of fibrin in the cerebrovasculature, is associated with inflammation in experimental autoimmune encephalomyelitis (EAE), the animal model of MS (Paterson et al. 1987). Thrombin is a principle protease that converts fibrinogen to fibrin in the coagulation cascade and, thus, thrombin may play an important role in MS. Recent studies have demonstrated that thrombin levels are not changed in the brain of patients with MS (Boven et al. 2003), but the endogenous thrombin inhibitors are significantly increased during EAE. The plasma thrombin-antithrombin III complex is increased before the onset

of EAE (Inaba et al. 2001). An abrupt and transient increase in the permeability of the blood–brain barrier occurs in animals with EAE at the time of early clinical manifestations of the disease (Juhler et al. 1984), which allows extravasation of plasma components into the brain parenchyma. In fact, the increased antithrombin III activity in the brain tissue has been observed at the peak of the disease, but with no changes of antithrombin III mRNA in the brain (Beilin et al. 2005; Chapman 2006). It was also shown that PN-1 is elevated in the brain before the clinical peak of EAE (Beilin et al. 2005). Therefore, attenuation of thrombin activity may be beneficial in MS (Chapman 2006).

PARs are significantly associated with inflammation in multiple physiological systems (Fiorucci and Distrutti 2003). The predominant expression of PAR₁ in oligodendrocytes (Wang et al. 2004) indicates the important role of PAR₁ in MS. In a recent report, it was shown that activation of PAR₂ in macrophages induces oligodendrocyte death in vitro, which is associated with proinflammatory gene expression (Noorbakhsh et al. 2006). In the myelin oligodendrocyte glycoprotein-induced EAE animal model in vivo, the PAR₂-mediated neuroinflammation is much more severe in wild-type mice than that in PAR₂ knockout mice. Moreover, the myelin loss is closely associated with macrophage infiltration/microglial activation during EAE, which is largely prevented in PAR₂ knockout mice. Notably, neurobehavioral analysis further indicates that PAR₂ contributes to severe neurological disability during EAE (Noorbakhsh et al. 2006). Therefore, thrombin and thrombin receptors are novel promising therapeutic targets in MS.

8.4.5 *Thrombin and HIV*

HIV infection is one of the rapidly spreading diseases. HIV-associated dementia is a neurodegenerative disease characterized by neuroinflammation and neuronal injury. During HIV encephalitis, the level of prothrombin/thrombin mRNA is significantly increased in the brain compared with control cases (Boven et al. 2003). Strong immunoreactivity of prothrombin is detected on both neurons and astrocytes in HIV cases. Similarly, the expression of PAR₁ is also upregulated at both mRNA and protein levels in human astrocytes with HIV infection (Boven et al. 2003). The abnormal expression of thrombin and PAR₁ implies that thrombin plays an important role in HIV-associated dementia. Indeed, thrombin has been shown to differentially regulate the expression of viral glycoprotein gp120 epitopes in H9 cells infected with HIV-1 LAI virus (Ling et al. 2004). Thrombin also increases the expression of 2F5 antigen, a neutralizing epitope of gp41. Importantly, thrombin significantly enhances HIV-induced cell fusion in a concentration-dependent manner. Recent studies demonstrated upregulation of PAR₂ on neurons in the brain tissue from patients with HIV-associated dementia, which might be induced by the proinflammatory cytokines TNF- α and IL-1 β . Moreover, activation of PAR-2 prevents neuronal cell death during HIV infection in vitro and in vivo (Noorbakhsh et al. 2005). PAR₁ has been shown to prevent cell death against brain

insults (Wang et al. 2007c), suggesting that PAR₁ might share the same neuroprotective effect as PAR₂ during HIV infection. However, the precise functions of thrombin and thrombin receptors in HIV-associated diseases still await future studies.

8.5 Conclusions

The data presented above clearly demonstrate that thrombin is a multifunctional molecule in the brain (Table 8.1). In the normal brain, thrombin regulates neural cell proliferation, differentiation, and development. Recent studies demonstrate the important role of thrombin and PAR₁ on endothelial progenitor cells. Thrombin and PAR₁ peptide agonist stimulate proliferation, differentiation, and migration of endothelial progenitor cells (Smadja et al. 2006; Tarzami et al. 2006). The neural progenitor cells play a very critical role in brain functioning and they are suggested to provide a potential therapeutic strategy for neurodegenerative disorders (Sailor et al. 2006; Taupin 2006). Thus, it will be of interest to investigate the effect of thrombin on neural progenitor cells in future.

Over the last decade, most studies paid much attention to the effects of thrombin in the brain under pathological conditions. Emerging evidence has revealed that thrombin at high concentrations contributes to the pathological processes in the brain during neurodegenerative diseases, including stroke, Alzheimer's disease, and Parkinson's disease. In contrast, low concentrations of thrombin can rescue cells to induce survival of neurons and astrocytes exposed to various brain insults. The neuroprotective effect of thrombin was observed in stroke, Alzheimer's disease, and Parkinson's disease. Interestingly, thrombin-mediated cell death and cell survival share initial signaling proteins. The opposite final consequences may result from differences in the amplitude as well as the duration of the signal. PARs have been documented to regulate thrombin-mediated neurodegeneration and neuroprotection in the brain, although the involvement of PARs in microglia functions is still controversial. PAR₁ has been shown to extensively mediate thrombin effects in the brain. In contrast, the functions of PAR₃ and PAR₄ in the brain are still largely unknown. It was reported that murine PAR₃ acts as a cofactor to facilitate thrombin binding to low-affinity murine PAR₄ (Nakanishi-Matsui et al. 2000). Recently, human PAR₃ was shown to be a co-receptor of PAR₁, that affects the G α_{13} coupling with PAR₁, and thus regulates PAR₁ signaling (McLaughlin et al. 2007). Understanding PAR₃ and PAR₄ signaling would largely improve our knowledge of thrombin functions in the brain. Overall, thrombin and PARs represent novel therapeutic targets for treating neurodegenerative disorders. Thrombin inhibitors, PAR agonists, and antagonists will be invaluable pharmacological tools as therapeutics. However, considering the poor selectivity and efficiency of presently available receptor antagonists, it is quite important to develop new selective and potent antagonists as drugs in future.

Recent studies demonstrate that neural PAR₁ can be cleaved and activated by brain trypsin IV/mesotrypsin (Grishina et al. 2005; Wang et al. 2006b; Knecht et al. 2007).

Table 8.1 Physiological and pathological roles of thrombin in the brain

	Effects	References
Physiological functions	Neural cell proliferation, differentiation and migration	Wang et al. (2002a), Suo et al. (2002a), Grand et al. (1989), Suidan et al. (1997)
	Neurite retraction	Zurn et al. (1988)
	Morphological changes	Mahajan et al. (2000), Pai et al. (2001)
	Synapse elimination	Liu et al. (1994), Lanuza et al. (2001, 2003)
	Synaptic plasticity; learning and memory	Gingrich et al. (2000), Almonte et al. (2007)
	Intracellular calcium mobilization	Wang et al. (2002a, 2004), Luo et al. (2005), Suo et al. (2002a)
Pathological functions	Release of proinflammatory cytokines and chemokines, and production of nitric oxide/neuroinflammation	Choi et al. (2003a, 2005); Suo et al. (2002a, 2003), Ryu et al. (2000), Katsuki et al. (2006), Wang et al. (2006a, 2007a), Fan et al. (2005), Boven et al. (2003)
	Microglial activation?	Suo et al. (2002b, 2003), Choi et al. (2003a, 2005), Lee et al. (2006), Hanisch et al. (2004)
	Brain edema	Lee et al. (1996), Hua et al. (2003b)
	Glial scar formation	Nicole et al. (2005)
	Glutamate release from astrocytes and NMDA receptor potentiation	Lee et al. (2007), Ramos-Mandujano et al. (2007), Gingrich et al. (2000)
	Increase in infarct volume and brain damage after ischemia	Junge et al. (2003), Olson et al. (2004), Striggow et al. (2000)
	Tau hyperphosphorylation, breakdown and aggregation	Suo et al. (2003), Arai et al. (2005)
	APP processing?	Igarashi et al. (1992), Chong et al. (1994), Davis-Salinas et al. (1994)
	Upregulation of apoE-4 expression	Mhatre et al. (2006)
	Apoptosis/cell death	Donovan et al. (1997), Rao et al. (2007), Striggow et al. (2000), Fujimoto et al. (2007), Xue et al. (2006), Suo et al. (2003b), Choi et al. (2003a, b, 2005)
	Differentially regulation of expression of glycoprotein epitopes of HIV, and increase in HIV-induced cell fusion	Ling et al. (2004)
	Cell survival/Neuroprotection	Vaughan et al. (1995), Pike et al. (1996), Wang et al. (2006a, 2007a)
	Brain tolerance	Xi et al. (1999, 2000), Hua et al. (2003a), Masada et al. (2000), Henrich-Noack et al. (2006a), Jiang et al. (2002)
	Increase in expression of SOCS3 and IL-10/Inhibition of prolonged inflammation	Yang et al. (2004), Kim et al. (2002)

Trypsin IV is predominantly expressed in the brain (Wiegand et al. 1993). In addition, several trypsin-like serine proteases have also been detected in the brain (Chen et al. 1995; Anisowicz et al. 1996; Gschwend et al. 1997; Sawada et al. 2000). The brain trypsin and trypsin-like serine proteases are potential endogenous agonists of PARs

in the brain (Luo et al. 2007). They are able to activate and/or inactivate PARs, modulating the thrombin signaling in the brain. Therefore, the interaction between thrombin and brain trypsin is important for thrombin functions in the brain.

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Chapter 9

The Role of Thrombin in Tumor Biology

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Abstract A large body of work supports the association of thrombosis and malignancy. Accumulating laboratory and clinical data point to the important role of thrombin in tumor biology. Thrombin is able to stimulate tumor adhesion and growth by direct tumor cell activation through membrane protease-activated receptors (PARs) or indirectly through platelet–tumor cell interactions and angiogenesis. Thrombin is able to enhance metastases by increasing tumor cell seeding into the circulation, by platelet-mediated tumor cell sequestration and protection from immune cells, and by stimulating tumor neoangiogenesis. In addition we hypothesize that thrombin may preserve dormant tumor cells in individuals.

9.1 Introduction

In 1865 Armand Trousseau recognized that cancer can cause thrombosis by observing “the white painful edema...in patients affected with...cancerous tumors,” which he called “phlegmasia alba dolens” and which he attributed to “conditions where blood acquired...tendency of spontaneous coagulation” (Trousseau 1865). Venous thromboembolism (VTE) is commonly observed in cancer patients with the incidence depending upon cancer type. Data from Medicare hospital discharge diagnoses between 1988 and 1990 in the USA revealed that ovarian cancer exhibited the highest incidence of cancer-related VTE (120 out of 10,000 patients, 1.2%) (Levitan et al. 1999). However, the true incidence of thrombosis in cancer can be much higher. For example, in a study of necropsies of 6,197 patients who died from cancer between 1960 and 1984, patients with ovarian cancer, cancer of the extrahepatic bile duct system, and cancer of the stomach had the highest prevalence of pulmonary embolism (35, 32, and 15%, respectively) (Svensen and Karwinski

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1989). Thrombosis can be the first clinical manifestation of cancer. Approximately 10% of patients who developed spontaneous thrombosis are found to develop cancer within several years (Prandoni et al. 1992). In addition, prognosis is worse for patients with cancer who develop thrombosis (Sorensen et al. 2000). Approximately, 40% of patients with thrombosis who were subsequently diagnosed with cancer had metastatic disease at presentation (Sorensen et al. 1998).

Tumor cells carry constitutively active tissue factor on their surface, which combines with factor VIIa to activate factor IX to IXa and X to Xa on the activated platelet surface, leading to thrombin production from prothrombin. The purpose of this chapter is to provide evidence that thrombin generated during thrombosis can augment tumor growth and metastases through multiple mechanisms. In addition, we hypothesize that thrombin may participate in the phenomenon of tumor cell dormancy.

9.2 Thrombin Can Stimulate Tumor Growth In Vivo

Thrombin can enhance tumor growth in vivo through its effect on tumor cell proliferation, angiogenesis, and tumor protection from immunologic surveillance.

9.2.1 Supporting Evidence

Thrombin is a potent mitogenic agent for mesenchymal cells (Carney et al. 1984; Chen and Buchanan 1975; Gospodarowicz et al. 1978). Thrombin can upregulate gene expression leading to oncogenesis and angiogenesis (see later). Functionally active thrombin was detected on postsurgical tumor specimens, including malignant melanoma, using affinity-ligand methodology (Zacharski et al. 1995). MV3, a highly aggressive human melanoma cell line enhanced plasma coagulation by recruiting factor Xa with conversion of prothrombin to thrombin (Geaquinto et al. 2008). Thrombin treatment caused a 413-fold increase in pulmonary metastases of CT26 colon and B16 melanoma cells when given intravenously to mice (Nierodzik et al. 1991). This was the first direct proof of thrombin enhancing tumor growth in vivo. Further studies with implanted subcutaneous mice models revealed that hirudin (a powerful thrombin inhibitor) decreased tumor size up to 11-fold (Hu et al. 2004).

9.2.2 Mechanisms of Thrombin–Tumor Interactions. Thrombin Can Directly Activate Tumor Cells Through Protease-Activated Receptors

Thrombin can directly activate tumor cell growth-stimulatory signals through proteinase-activated receptors (PARs), leading to downstream mitogenic signaling

events. Thrombin acts on its G-protein-coupled seven transmembrane spanning receptors PAR₁, PAR₃, and PAR₄ by cleavage of their N-terminal ends. This exposes a tethered ligand that binds to the second extracellular loop of PAR₁ and activates the receptor. Treatment of tumor with the thrombin receptor activation peptide (PAR-AP) enhances growth of pulmonary metastases 17- to 320-fold for murine tumor cell lines B16F10 and CT26 in the absence of adhesion to platelets, pointing to thrombin as having a direct effect on tumor cells (Nierodzik et al. 1996). PAR₁ has been identified as a candidate oncogene by gene expression profiling using a cDNA library for genes associated with Rho-mediated signaling pathways, loss of anchorage independence, serum-dependent growth, focus-forming activity, and transformation of an NIH3T3 cell line (Martin et al. 2001). Thrombin upregulates nuclear orphan receptor TR3, a sensitive marker for PAR signaling, in MDA-MB-231 human breast cancer cells in vitro (Versteeg et al. 2008). In a recent study, PAR₁ expression in invasive breast carcinoma was proven to be critical for tumor growth in vivo. When PAR₁-deficient and control MDA-MB-231 breast cancer cells were implanted into the left and right mammary fat pads, respectively, of 6- to 8-week-old female immunodeficient mice, PAR₁-deficient cells revealed a significant reduction in tumor growth compared with control cells followed over a 6-week period (Arora et al. 2008).

Indirect Action Through Platelet-Tumor Interactions: Thrombin, a potent platelet agonist, can augment tumor growth indirectly by activating platelets and enhancing platelet-tumor cell interaction. Platelets can secrete tumor cell growth and angiogenesis factors, such as platelet-derived growth factor (PDGF) (Kepner and Lipton 1981), vascular endothelial growth factor (VEGF) (Mohle et al. 1997), and angiopoietin-1 (Ang-1) (Li et al. 2001). Platelets produce lysophosphatidic acid (LPA) that induces tumor cell proliferation by binding its LPA1 receptor on breast (MDA-BO2) and ovarian (CHO) cancer cells (Boucharaba et al. 2004).

Thrombin Augments Tumor Growth Through Induction of Angiogenesis: Most tumors require a blood supply once they reach 5 mm in size. Therefore, angiogenesis is critical for tumor growth (see chapter by Tsopanoglou and Maragoudakis in this volume). Thrombin increased angiogenesis two- to threefold when applied to chorioallantoic chick membrane. Thrombin upregulated VEGF-A and Ang-2. Thrombin-induced angiogenesis was blocked by hirudin, soluble decoys for VEGF-A (VEGFR2-Fc), and angiopoietin (Tie2-Fc) receptors. Angiogenesis was initiated by PAR₁-specific peptide (SFLLRN) that led to downstream signaling through protein kinase C, MAP kinase, and PI-3kinase (Caunt et al. 2003).

Thrombin stimulates angiogenesis by inducing vascular endothelial growth factor (VEGF-A) production by tumor cells (Huang et al. 2000; Ollivier et al. 2000). Thrombin induces vascular endothelial growth factor receptor VEGFR2 expression (Tsopanoglou and Maragoudakis 1999) and angiopoietin-2 production (Ang-2) in endothelial cells (Huang et al. 2002). In addition, thrombin triggers endothelial tube formation in a matrigel membrane system (Haralabopoulos et al. 1997). Thrombin enhances VEGF-A (Mohle et al. 1997) and Ang-1 secretion by platelets (Li et al. 2001).

Thrombin and chemokine growth-regulated oncogene-a (GRO-a) in angiogenesis: The growth-regulated oncogene-a (GRO-a) is a CXC chemokine with oncogenic

potential. It has an important role in blood vessel formation and maintenance of wound repair (Devalaraja et al. 2000). *GRO-a* binds to CXCR2 receptor on endothelial cells and attracts neutrophils (Strieter et al. 2005). In addition, *GRO-a* enhances growth, chemotaxis, and metastasis of several tumor cell lines (Loukinova et al. 2000; Keane et al. 2004). Importantly, *GRO-a*-augmented metastasis is linked to increased angiogenesis (Baggiolini et al. 1994; Loukinova et al. 2000; Keane et al. 2004). Thrombin significantly upregulates secretion of *GRO-a* from tumor cells as well as from human umbilical vein endothelial cells (HUVECs). *GRO-a*, like thrombin, stimulates neoangiogenesis, endothelial cord formation, and endothelial cell growth. *GRO-a*, like thrombin, activates an identical series of five proangiogenic genes. Anti-*GRO-a* antibody or small interfering RNA (siRNA) *GRO-a* knockdown (KD) negates the 2.7-fold thrombin-induced angiogenesis and 1.5-fold upregulation of endothelial cord formation. In addition, anti-*GRO-a* antibody inhibits chemotaxis and tumor cell growth. Thrombin-augmented tumor growth, angiogenesis, metastasis, and upregulation of VEGF and Ang-2 are inhibited in wild-type mice using *GRO-a* knockdown (KD) 4T1 breast tumor cells. Thrombin and PAR₁ receptor activation peptide (PAR-AP) as well as *GRO-a* all significantly stimulated vascular regulatory proteins and growth factors: matrix metalloproteinase (MMP)-1, MMP-2, VEGF, Ang-2, CD31, and receptors VEGFR2 and CXCR2 in HUVECs. These experiments suggest a critical role for *GRO-a* in thrombin-induced angiogenesis (Caunt et al. 2006).

Thrombin and Platelets in Angiogenesis: Although platelets can release both angiogenic (VEGF-A, bFGF, ANG-1, PDGF) and antiangiogenic growth factors (thrombospondin, PF-4, endostatin), the thrombin-induced platelet activation shifts the balance in favor of angiogenesis (Brill et al. 2004, 2005).

9.3 Thrombin and Metastases

Thrombin can promote and enhance metastases through its effect on tumor cells, tumor cell interactions with platelets, endothelial cells, extracellular matrix and through stimulation of angiogenesis.

9.3.1 Supporting Evidence

Thrombin increased experimental pulmonary metastases (CT26, B16F1, B16F10 tumor cells) in mice by 10- to 156-fold (Nierodzik et al. 1992). These experiments demonstrated a direct connection between thrombin and metastases in vivo. Hirudin, a direct acting thrombin inhibitor, decreased metastases and increased survival in mice. Hirudin treatment led to a 15- to 32-fold reduction in circulating tumor cells and more than 2.2-fold reduction in spontaneous pulmonary metastases in vivo (Hu et al. 2004). Overexpression of thrombin protease-activated receptors

PAR₁ in B16 melanoma cells caused a fivefold increase in pulmonary metastasis in mice (Nierodzik et al. 1998). A single dose of human antihemophilic factor (factor VIII) significantly increased B16F10 lung metastases in mice with hemophilia A. Lepirudin, a potent direct thrombin inhibitor, significantly decreased lung seeding pointing to an important role of thrombin in pulmonary metastases even without presence of factor VIII (Langer et al. 2006). Plasma levels of prothrombin fragments F1 + 2 were found to correlate with lymph node metastases and clinical stage in patients with gastric cancer (Kwon et al. 2008). Argatroban, a potent thrombin inhibitor, decreased breast cancer metastasis via osteopontin-dependent and osteopontin-independent mechanisms (Schulze et al. 2008). In a recent study, MDA-MB-231 human breast cancer cells were intravenously injected into the tail vein of SCID mice. Mab-5G9, an antibody against TF-VIIa-Xa consistently reduced the load of pulmonary MDA-MB-231 human breast cancer cells in mice after 24 h (Versteeg et al. 2008). Twist, which is upregulated by thrombin (Hu et al. 2008), is required for spontaneous metastases in a variant 4T1 murine breast cancer (Yang et al. 2004). A recent large meta-analysis based on data from four major studies on anticoagulation in cancer patients found that low molecular weight heparins improved overall survival in cancer patients, even in those with advanced disease (Lazo-Langner et al. 2007).

9.3.2 Mechanisms of Thrombin Promoting Metastases. Activation of PAR₁

In a recent study, thrombin activation of PAR₁ triggered activation of epidermal growth factor receptor (EGFR) and ErbB2/HER2 receptor in invasive breast carcinoma, but not in normal mammary cells in mice. This led to activation of extracellular signal-regulated kinase-1 and -2 signaling and stimulated MDA-MB-231 breast carcinoma cell invasion. In addition, thrombin-PAR₁ signaling through Galpha(i/o) and metalloproteinase activity was found to be critical for ErbB-mediated cell invasion (Arora et al. 2008).

Tumor Cell-Platelet Interaction: Thrombin is a potent platelet activator. Platelets were found to play an important role in metastases in animal models (Gasic et al. 1968; Camerer et al. 2004). Studies demonstrated metastatic tumor cell emboli surrounded by platelets. It has been proposed that platelets may protect tumor cells in the circulation and increase tumor cell survival (Jones et al. 1971). It has been demonstrated that <2% of intravenously injected tumor cells survive in the mouse circulation after 24 h. Platelets protect tumor cells from natural killer (NK) attack (Nieswandt et al. 1999; Palumbo et al. 2005). In addition, activated platelets secrete many metastasis-promoting growth factors, such as VEGF (Mohle et al. 1997), PDGF (Kepner and Lipton 1981), and Ang-1 (Li et al. 2001). Platelet-derived lysophosphatidic acid (LPA) enhances bone metastasis by inducing secretion of potent osteoclast stimulators such as interleukin IL-8 and IL-6 (Boucharaba et al. 2004).

Tumor Cells–Platelet Adhesion: The specific fibronectin peptide Arg-Gly-Asp-Ser (RGDS domain), von Willebrand factor (vWF), platelet P-selectin, and platelet glycoprotein (GP) IIb/IIIa (integrin α IIb β 3) and thrombin are important for tumor cells' adhesion to platelets. Pentapeptide GRGDS (H-Gly-Arg-Gly-Asp-Ser-OH) reduced B16F10 melanoma pulmonary metastases in mice by 97% (Humphries et al. 1988). Anti-vWF antibody reduced experimentally induced metastasis by 65% in mice (Karpatkin et al. 1988). P-selectin-deficient mice have slower tumor growth in vivo and reduced metastasis in association with the lack of platelet–tumor aggregates (Kim et al. 1998).

P-selectin is important for tumor cell tethering and rolling leading to subsequent tumor cell–platelet adhesion caused by GPIIb-IIIa and vWF through an RGD-mediated process (McCarthy et al. 2000). GPIIb-IIIa is required for soluble fibrin monomer enhanced platelet–tumor cell adherence in vitro and in vivo (Biggerstaff et al. 1999). Thrombin treatment of platelets increased tumor cell–platelet adhesion two- to fourfold in six tumor cell lines (HM54 hamster melanoma; human HCT8 colon cancer and SK-Mel-28 melanoma; and murine B16a melanoma, KLN205 squamous cell, and CT26 colon cancer) (Nierodzik et al. 1991). In vivo experiments in which both thrombin and tumor cells were injected into mice increased experimental pulmonary metastases 4–413-fold in two tumor cell lines (CT26 and B16a) (Nierodzik et al. 1991). Two possible mechanisms are proposed: (1) thrombin increases exposure of platelet GPIIb-IIIa on the platelet or tumor cell surface, (2) thrombin enhances the release of platelet fibronectin and vWF onto the platelet surface. Thrombin-activated M3 Dau melanoma cells develop a *GPIIb-IIIa-like* receptor on their surface (which is inhibited by anti-GPIIb-IIIa and RGDS) (Boukerche et al. 1989; McGregor et al. 1989; Nierodzik et al. 1992). These cells do not react with Glanzmann thrombasthenia platelets (congenital absence of GPIIb-IIIa) and, if pretreated with anti-GPIIb-IIIa antibody, do not grow in nude mice (Boukerche et al. 1989). The GPIIb-IIIa platelet receptor was found to have a universal presence in tumor cell lines originating from many organs: blood, lung, liver, bladder, breast, prostate, colon, skin, kidney, and cervix.

Thrombin can augment tumor cell–platelet adhesion through protease-activated receptor (PAR₁), a G-protein-coupled receptor ubiquitously found on 11 tumor cell lines examined either by immunoblot or by RT-PCR. Importantly, 7 of 11 lines responded to the PAR₁ thrombin receptor activation peptide (PAR-AP), by a two- to threefold enhanced adhesion to platelets (Nierodzik et al. 1996). In addition, genetically manipulated mouse models support the important role of platelets and fibrinogen in metastases. In a B16 melanoma model using *NF-E2/-* knockout mice (no platelets), *PAR-4/-* knockout mice (no major platelet thrombin receptor), and *Fib/-* knockout mice (no fibrinogen) experimental pulmonary metastases were greatly decreased (Camerer et al. 2004).

Tumor Cell–Endothelial Adhesion: Thrombin treatment of SKMel-28 and HM29 tumor cells caused a 2.3-fold increase in their adhesion to bovine endothelium (Klepfish et al. 1993). Hela or HT29 tumor cells were found to have greater adhesion to endothelial cells in vitro in the presence of both platelets and thrombin than in the presence of platelets or thrombin alone (Helland et al. 1997). Thrombin

treatment of human melanoma 397 cells leads to a 2.2-fold increase in the adhesion of human melanoma cells to endothelial cells (Dardik et al. 1998).

Other Tumor Cell–Endothelial Interactions: Thrombin is able to disrupt endothelial cell junctions maintained by vascular endothelial cadherin (VE cadherin) and β -catenin (Konstantoulaki et al. 2003). It had been shown that an antibody against VE cadherin decreased tumor growth (Liao et al. 2000). Thrombin stimulated expression and association of beta1-integrin with matrix metalloproteinase 9 (MMP-9) on the cell surface of a human osteosarcoma, a process that promoted tumor cell invasion (Radjabi et al. 2008).

9.3.3 *Thrombin and Metastasis: A Proposed Mechanism*

We propose the mechanism of thrombin-stimulated metastases as follows: (a) *Initiation.* Tumor cell surface tissue factor combines with factor VIIa to activate factor IX to IXa and X to Xa on the activated platelet surface. Factor Xa's action results in the initial weak conversion of prothrombin to thrombin. Thrombin disrupts endothelial cell junctions and stimulates migration of tumor cells from the extravascular space into the vasculature. Thrombin activates P selectin expression on endothelial cells. (b) *Weak interaction.* P-selectin-expressing endothelial cells and activated platelets weakly bind to tumor cells containing the P-selectin ligand receptor. (c) *Amplification.* Platelets produce a catalytic surface for the coagulation cascade, which enhances thrombin generation that activates more platelets. (d) *Strong interaction.* Increased thrombin generation and platelet activation lead to strong binding between platelets and tumor cells through platelet integrin IIb-III, tumor integrins, vWF, fibronectin, and other RGDS ligands. (e) *Propagation.* This leads to angiogenesis via thrombin-enhanced secretion of VEGF and GRO- α from tumor cells; PDGF, VEGF, and Ang-1 from platelets; and Ang-2 and VEGFR2 from endothelial cells. Platelets protect tumor cells from NK cells and promote further emboli downstream, leading to ischemic vessel wall damage exposing subendothelial basement membrane and matrix. Tumor cells and platelets bind more avidly to subendothelial basement membrane and matrix. Tumor emboli lead to tumor extravasation into the parenchyma, neoangiogenesis, and metastases. Thus, growing tumors initiate a *vicious cycle* in which greater tumor burden supplies greater thrombin, which leads to a stronger platelet–tumor interaction (Nierodzik and Karparkin 2006).

9.4 Thrombin and Tumor Cell Dormancy

9.4.1 *Evidence for Dormancy*

A growing body of evidence points to a phenomenon of tumor cell dormancy. Breast cancer recurrences have been observed decades after removal of primary

tumor (Stein and Litman 2006). Early stage melanoma is known to remain dormant for many years, after which it may convert to a highly aggressive tumor (Bayko et al. 1998). Melanoma has been documented in two kidney transplant recipients 16 years after surgical removal in the donor (MacKie et al. 2003). Chronic lymphatic leukemia (CLL) clones are detected in asymptomatic elderly men as well as siblings of patients with CLL (Rawstron et al. 2002). Patients with monoclonal gammopathy of undetermined significance (MGUS) have an approximately 1.0–1.5% per year rate of progression to multiple myeloma (Kyle 1997). Autopsies of individuals who died from nonmalignant disease often reveal early stage cancers. For example, thyroid cancer was detected in 38% of 101 autopsies (Harach et al. 1985), breast cancer in 20% of 110 autopsies (Nielsen et al. 1987), and prostate cancer in up to 30% of autopsies (Konety et al. 2005).

9.4.2 *The Possible Role of Thrombin*

Shulman and Lindmarker (2000) presented 6-year follow-up data on 854 patients who had been treated with coumarins for deep vein thrombosis. Patients treated for 6 months with coumarin developed significantly fewer tumors than patients treated for 6 weeks [odds ratio of 1.6 (95% CI 1.1–2.4; $p = 0.02$)]. The Northwick Park Heart Study (Miller et al. 2004) prospectively evaluated hypercoagulability once a year for 4 years with follow-up of 11 years in 3,052 men with no clinical evidence of malignancy. Hypercoagulability was defined as two yearly detections of increased prothrombin activation fragments 1 + 2 and fibrinopeptide A. Cancer death rates were higher in people with hypercoagulability (11.3% vs. 5.1%, with a relative risk of 2.2 ($p = 0.015$)). Hypercoagulability correlated with a more aggressive cancer. We hypothesize that low-dose thrombin can contribute to preserving tumor dormancy by preventing tumor eradication. Tumor growth *awakening* occurs with increasing concentration of thrombin exposure. Additional factors, such as immune surveillance and angiogenesis, may contribute to this process.

The earlier reviewed experimental animal data on role of thrombin in tumor biology, as well as insightful observations of Shulman and Lindmarker (2000), provide strong support for the use of antithrombin-based therapy for the adjuvant treatment of patients with cancer.

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Chapter 10

The Role of Thrombin and its Receptors in Epithelial Malignancies: Lessons from a Transgenic Mouse Model and Transcriptional Regulation

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Abstract The otherwise well-orchestrated epithelial sheets are disrupted when they acquire the ability to overexpress the prototype mammalian thrombin receptor, *human protease-activated receptor-1 (hPar1)*. This is exhibited by down-regulation of cell–cell contacts and alterations in cell–matrix interactions. The notion that *hPar1* is one of a series of genes that is part of a malignant program stems from studies indicating that *hPar1* expression directly correlates with tumor metastasis and the time-limited physiological invasion of the placenta to the uterus decidua. Our transgenic mouse model of tissue-targeted *hPar1* overexpression in the mammary glands exhibits a phenotype of hyperplasia, characterized by a dense network of ductal side branching and accelerated proliferation. The transgenic mammary glands exhibit increased levels of *wnt-4* and *-7b*, and the striking stabilization of β -catenin. This novel association between *hPar1* and nuclear β -catenin may provide a key determinant in the molecular pathway of *hPar1* oncogenicity. While studying the properties of *hPar1* in tumor biology we demonstrated its role as a survival factor that protects cells from undergoing apoptosis. Withdrawal of the *hPar1* gene leads to selective apoptosis especially in young sprouting blood vessels, whereas mature vessels remain unaffected. We also provide evidence showing that *hPar1* gene overexpression in tumors stems from enhanced transcriptional activity. This is evaluated on the basis of elicited run-on transcription rate in highly metastatic vs. low metastatic cells (on a background of equal stability rates). Indeed, we have shown that the transcription factor *Egr-1* induced *hPar1* gene overexpression in prostate cancer. In addition, the tumor suppressor gene p53 also acts on *hPar1* as one of its target genes, regulating its level of expression in the context of a given tumor. It still remains to identify specific motifs within *hPar1* promoter that bind to transcription factors and/or tumor suppressor genes, critically involved in *hPar1* transcription.

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10.1 Morphogenesis of Epithelia Sheets

Most human cancers arise from epithelial sheets. The highly ordered, well-orchestrated epithelial tissue is anchored to basement membranes, displaying cell–cell contact and polarized cell morphology. Under normal conditions, the epithelial sheets serve as natural barriers throughout the body, maintaining highly regulated events necessary for proper proliferation, survival, and differentiation (O'Brien et al. 2002). Once this intact architecture is disrupted, epithelial tumor pathogenesis is initiated. Indeed, for decades architectural features served as a diagnostic tool for classifying epithelial tumors. Today, accumulating evidences point to a clear relationship between defined molecular abnormality and histological phenotype. For example, invasive lobular carcinoma is typically associated with the loss of a tumor suppressor gene, *CDH1*, which encodes E-cadherin and is important for the proper maintenance of cell–cell contact (Berx et al. 1996). Another type of breast cancer, ductal carcinoma in situ (DCIS), exhibits amplified levels of the oncogene *HER2/Neu*, which encodes the epidermal growth factor (EGF) family member erbB2 (van de Vijver et al. 1988). Still, for the most part, little is known about the genotypic abnormalities associated with the changes in cancer phenotype. A better understanding of the molecular mechanism underlying morphogenetic alterations may be supplied by insight into the progression of carcinoma, which, in turn, may contribute to the discovery of diagnostic tools and cancer therapeutic medicaments. For this purpose we chose to use primary human tumor tissues and mouse models of pathological epithelia to evaluate gene related morphogenesis.

10.2 PAR₁ Overexpression Directly Correlates with Metastatic Potential: Lessons from Malignant and Physiological Invasion Processes

Protease-activated receptor1 (PAR₁), the cellular counterpart of thrombin, the major coagulation serine protease, is a G protein-coupled receptor (GPCR). PAR₁ serves as a prototype of the mammalian PARs, a family comprising four members. PAR₁ activation involves the release of N-terminal peptide and the exposure of an otherwise hindered ligand, providing a unique mode of activation and a general paradigm for the entire PAR family. Unlike most cellular receptors, PARs do not require the traditional ligand–receptor complex formation for activation. Instead, these receptors serve as substrates for proteolytic digestion yielding irreversible activated receptors (Coughlin 2000; Rasmussen et al. 1991; Vu et al. 1991). The notion that *hPar1* is one of a series of genes that is part of a malignant program stems from studies indicating that *hPar1* expression directly correlates with tumor metastasis (Even-Ram et al. 1998; Henrikson et al. 1999; Nierodzik et al. 1998; Yin et al. 2003; Salah et al. 2005; Grisaru-Granovsky et al. 2005; Fig. 10.1). Analyses of tumor biopsy specimens originating from an array of tumor epithelia,

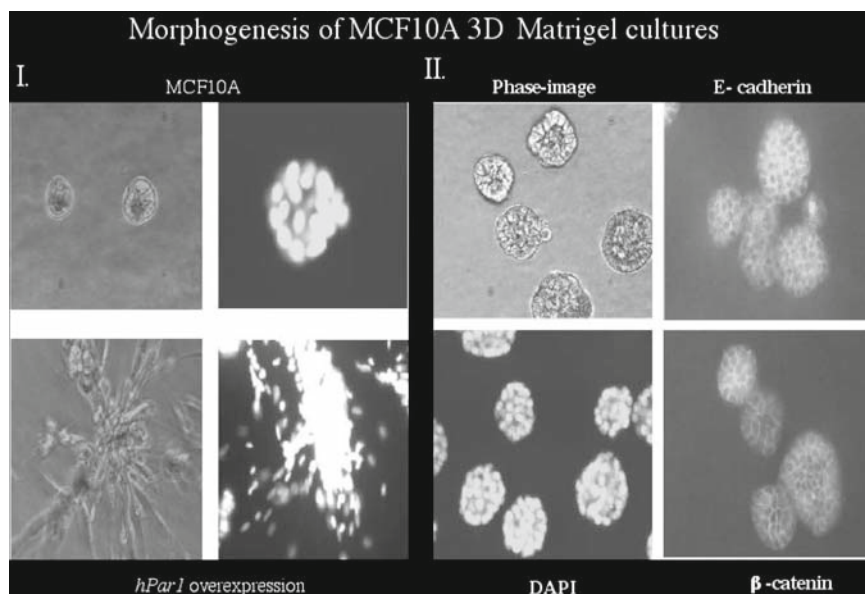


Fig. 10.1 Morphogenesis of MCF10A breast cancer cells grown in a Matrigel milieu. (I) *Upper section*. Intact spheroids exhibiting cell–cell and cell–matrix interactions are observed in MCF10A cells plated on a three-dimensional Matrigel layer. *Bottom*. When *hPar1* viral vector-infected MCF10A cells are plated in a Matrigel layer an interrupted structure with many invading villi is observed. (II) DAPI staining for nuclei localization is observed. Cell–cell contacts are shown via β -catenin staining

as well as a panel of differentially metastatic cell lines, have pointed out the direct correlation between levels of *hPar1* expression and tumor progression. This is based on a careful survey of a panel of mammary carcinoma cell lines showing high levels of *hPar1* expression in aggressive metastatic breast cancer cells (e.g., MDA-435, MCF10AT3B), somewhat lower levels in moderately metastatic breast cancer cell lines (e.g., MDA-231, MCF10T), and very low levels to nearly none in nonmetastatic breast cancer cells (e.g., MCF-7, ZR-75, and MCF10A). In situ hybridization analyses performed on paraffin-embedded sections of archival biopsy specimens have shown a differential pattern of highly specific staining localized to the epithelial cells of invasive ductal carcinoma (IDC and DCIS), while very little to no staining was observed in premalignant atypical intraductal hyperplasia (AIDH) or normal mammary sections obtained from reduction mammoplasty. Likewise, a similar approach was undertaken in prostate malignancies and in ovarian and endometrium cancer progression, underscoring the central role of PAR₁ in epithelial tumor progression (Even-Ram et al. 1998).

PAR₁ expression does not merely correlate with tumor progression, but appears likely to play an active role during metastatic breast carcinoma cell invasion. This is based on the fact that introduction of PAR₁ antisense or SiRNA (small interfering RNA) into aggressively metastatic MDA-435 or other metastatic cells that highly

express *hPar1* (e.g., C1-1 and Du-145 prostate cancer cells) (Salah et al. 2005) markedly attenuated the ability of the cells to migrate through Matrigel (a reconstituted basement membrane)-coated filters in vitro.

10.3 Placenta Physiological Invasion

The development of placental tissue is a highly regulated process that begins as early as fertilization and continues throughout pregnancy. This process is essential for the proper maintenance of normal fetal growth, development, and safeguarding of normal pregnancy. The progenitor villous trophoblast is the “stem cell” of the placenta, differentiating along two main pathways: the invasive extravillous trophoblast (EVT) and the syncytiotrophoblast. The invasive extravillous trophoblast is the part that is responsible for anchorage of the placenta to the decidua and the myometrium (Fisher and Damsky 1993; Cross et al. 1994; Cartwright et al. 2002; Bischof et al. 2006; Carter et al. 2006). Cytotrophoblastic cells (CTBs) are derived from the trophoectodermal cells of the blastocyst and represent a heterogeneous population. Once the placental villi are formed, some CTBs of the anchoring villi which contact the uterine wall acquire a transiently invasive phenotype and occupy the decidualized endometrium, while the CTB of the floating villi in the extravillous space remain attached to the villous basement membrane (Pijnenborg et al. 1980). These highly motile and invasive extravillous CTB, also referred as intermediate trophoblast, are found as cytokeratin-positive cells in the deciduas, the intima of the uterine blood vessels, and the proximal third of the myometrium (Pijnenborg et al. 1981; Vercruysse et al. 2006). The invasive behavior of trophoblasts shows striking similarities as well as differences to tumor invasion (Lala et al. 2002).

The resemblance between trophoblastic and transformed cells prompted the study of oncogenes in the human placenta. EGF receptors are predominantly expressed on the villous trophoblast and not on the invasive EVTs (Jokhi et al. 1993; Bass et al. 1994). The product of *c-fli* proto-oncogene (an *fms*-like tyrosine kinase), the receptor of the angiogenic factor vascular endothelial growth factor (VEGF), is expressed in both the villous and the EVT. *C-sis* that encodes the beta chain of the platelet-derived growth factor (PDGF) and *c-myc* oncogenes are co-expressed in the cytotrophoblast (Roncalli et al. 1994; Osterlund et al. 1996). The p53 tumor suppressor gene has also been considered as a potential regulator of trophoblast invasion since all types (e.g., villous and extravillous) express the wild-type p53 form for the appropriate balance maintained between oncogenes and tumor-suppressor genes, while the choriocarcinoma lines express a mutated p53 form (Bishop 1991). The normal implantation process thus provides an attractive model to explore basic issues in a physiological invasive gene program. The use of small tissue fragments of the human placenta, the villous explants, has been found to be a suitable model for studying trophoblast invasion since they ideally represent in vitro, and maintain most closely, the entire context of the in vivo environment of these cells (Genbacev et al. 1993, 2000; Vicovac et al. 1995; Aplin et al. 1998;

Genbacev and Miller 2000). We hypothesized that genes that are part of an invasive program are highly expressed during the placenta physiological invasion period and are turned off immediately thereafter. For this purpose, levels of *hPar1* gene expression were measured in trophoblasts from early pregnancy placentas, between 6 and 12 weeks of gestation, obtained from legal abortion products. Indeed, our data from placenta tissue sample analysis showed a spatial distribution of PAR₁. High levels were found exclusively during the time-limited invasion period, between 6 and 10 weeks of gestation, and were completely shut off thereafter. Likewise, high and unrestricted levels of PAR₁ were detected in the age-matched pathological samples of molar placenta (Even-Ram et al. 2003). Uncontrolled trophoblast proliferation/growth results in gestational trophoblastic disease (GTD). GTD encompasses a number of diverse lesions such as complete/partial hydatiform mole, invasive mole, choriocarcinoma, and epitheloid trophoblastic disease. Choriocarcinoma is the most severe form and the tumor cell resembling primitive trophoblasts of the previllous stage development arrested in specific stages of differentiation. The complete hydatiform mole consists of uncontrolled proliferation of villous trophoblasts which may progress to invade the myometrium and the blood vessels. Complete moles have potential for local invasion (15–33%) and dissemination (4–9%). Indeed, the above-described GTD exhibits high levels of *hPar1* gene expression and highlight the part of the gene involved in invasion.

Summary. While *hPar1* is overexpressed in malignant tumors, *hPar1* levels in the physiological invasion process are high in a time-limited fashion, during the first trimester of pregnancy, and are subsequently shut off. What are the regulatory processes that control the initiation of signals that shut off the gene when the invasion process is over remains an intriguing question.

10.4 Transgenic Mice of *hPar1* Targeted to Overexpress in the Mammary Gland Tissue

To gain further insight into the causal relationship between *hPar1* expression, breast tumor formation, and mammary gland developmental morphogenesis, we have established a line of mice carrying MMTV-long terminal repeat (LTR)-SV40-driven *hPar1* designed to overexpress in the mammary glands. While mammary tissues can be used to study discrete developmental remodeling aspects of the breast, they also provide an opportunity to dissect the contribution of individual genes in normal and malignant mammopoiesis.

To determine the effect of *hPar1* expression on mammary gland morphogenesis, histological preparations of mammary tissues from various developmental stages were analyzed. Whole mounts of mammary glands from wild-type mice were compared with age-matched transgenic littermates. The mammary glands of *hPar1*-tg mice showed increased branching with moderate to marked lobulo-alveolar development. During normal mammary growth, rudiments of the ducts formed at birth grow slowly until onset of puberty, when terminal end buds form and

ductal elongation and bifurcation begin. Normally, the degree of ductal side branching and the number of terminal end buds in the virgin wild-type animals increase with age (until ~6 weeks of age). Overall, in *hPar1* overexpressing glands a consistently greater ductal complexity was observed as compared with their age-matched wild-type counterparts. During pregnancy when the estrous hormonal system is high, ductal side-branching is even more striking, exhibiting high levels of ductal network complexity and increased alveolar buds in the *hPar1*-overexpressing mice (Yin et al. 2006).

10.5 *Wnt-4* and *wnt-7b* are Overexpressed in *hPar1*-tg Mammary Glands

Since one of the main molecular mechanisms underlying mammary gland ductal side branching suggests an essential role for *Wnt-4*, we screened levels of mouse *wnts* in normal vs. transgenic mice mammary glands. *Wnt* proteins are soluble glycoproteins which initiate cell signaling through binding to receptor complexes composed of *Frizzled* proteins and LDL receptor-related protein (LRP). Indeed, we found specifically elevated levels of *wnt-4* and *-7b* in the *hPar1*-tg mammary glands. No change was detected in any of *wnt-2*, *-5a*, or *-6*, or in *wnt-7a*, *-5b*, or *-10b*. Normally, virgin wild-type mice express low levels of *wnt-4*, which increase slightly during pregnancy. In comparison, mice overexpressing *hPar1* have higher levels of *wnt-4* in virgin mammary glands, and a significantly greater level of enhancement is observed throughout pregnancy. One of the target transcriptional activation genes of the *wnt* pathway is *cyclin D1*, the major G1 cyclin expressed in mammary epithelial cells (Shtutman et al. 1999; Yu et al. 2001). In support of this, we found increased levels of cyclin D1 expression in *hPar1*-overexpressing mammary glands as compared with wild-type counterparts.

10.6 β -Catenin Stabilization by *hPar1*

The signaling pathway downstream of *wnt* assigns β -catenin stabilization as a major part. Indeed, western blot analyses of wild-type- and *hPar1*-overexpressing age-matched counterparts showed a striking accumulation of β -catenin in the mammary gland tissues of both virgin and pregnant *hPar1* transgenic mice as compared with minimal levels in the mammary tissues of wild-type controls (Fig. 10.2). The mammary gland tissues showed marked nuclear localization of β -catenin in the *hPar1*-overexpressing mice, in which it affects target genes downstream. This holds true also in a panel of transformed epithelial cells showing accumulated β -catenin, in parallel to the transgenic mouse tissue (for

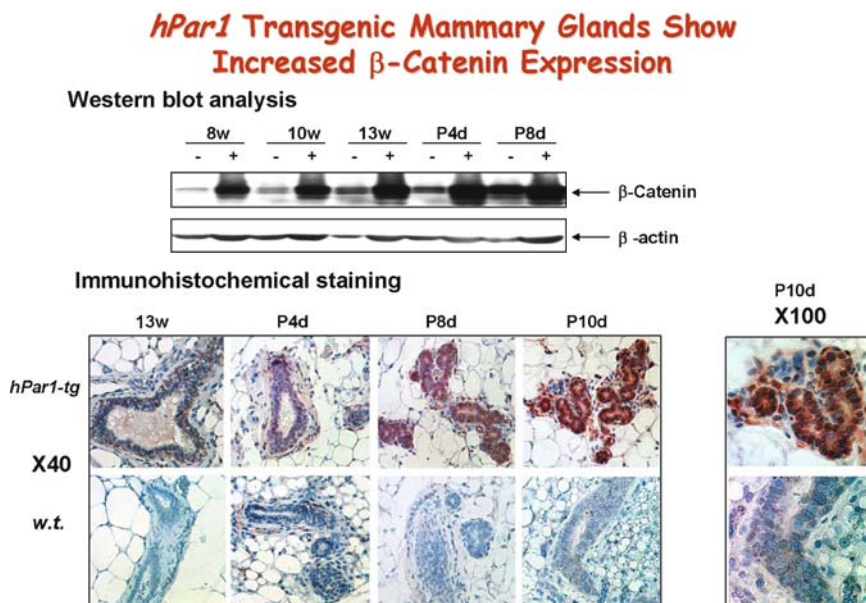


Fig. 10.2 (a) Western blot analysis of β -catenin expression in *hPar1-tg* mammary glands at different developmental stages compared with age-matched wt mammary glands. (b). Immunohistochemical staining shows β -catenin localization in the mammary glands of *hPar-tg* and wt mice. At various days of development of virgin and pregnant mammary glands, nuclear β -catenin staining is observed in the *hPar1-tg* mice but not in the wt age-matched mice (see *Color Plates*)

example, the colorectal cancer cell lines HT-29 and HCT-116 cells). While these colon cancer cell lines are mutated (HT-29, truncated APC; adenomatous polyposis coli or mutated β -catenin at amino acid 45 in HCT116) on the background of β -catenin pathway, a control experiment in cells exhibiting intact β -catenin was carried out. RKO is a colorectal cancer cell line transformed on the basis of gene instability (Brattain et al. 1984). This cell line displays microsatellite instability for hypermethylation of the hMLH1 promoter, but expresses wild-type APC, β -catenin, and p53 (Eshleman et al. 1996; da Costa et al. 1999). Indeed, following PAR_1 activation and pretreatment of cells with MG132 (an inhibitor of the proteasomal system), enhanced nuclear β -catenin levels are observed. Therefore *hPar1* gene expression and activation are directly involved with the accumulation of β -catenin, a major regulator of the oncogenic pathways. We propose that *hPar1* expression and activation elicit primarily the striking stabilization of β -catenin and *wnt* expression. Our studies nonetheless identify *hPar1* gene as a potent target for cancer therapy because neutralization of the gene may effectively inhibit initiation of oncogenicity via *wnt* generation and β -catenin stabilization that ultimately enters the nuclei to affect an array of target genes downstream.

10.7 *hPar1* Acts as a Survival Factor While Promoting Tumor Progression

Less attention has been paid to the fate of a tumor when *hPar1* is silenced. We analyzed *hPar1* antisense clones, which exhibit low levels of PAR₁, attenuated cell proliferation and invasion in vitro, and tumor formation in vivo. When injected into mice, human *Par1* antisense clones resulted in very few and only occasional small tumors, whereas vascularized advanced tumors were observed in A375SM cells. The small tumors derived from the antisense clones expressed high levels of apoptotic enzymes such as active caspase-3, had many apoptotic cells, measured by TUNEL staining, and markedly reduced proliferating cell nuclear antigen levels (PCNA),

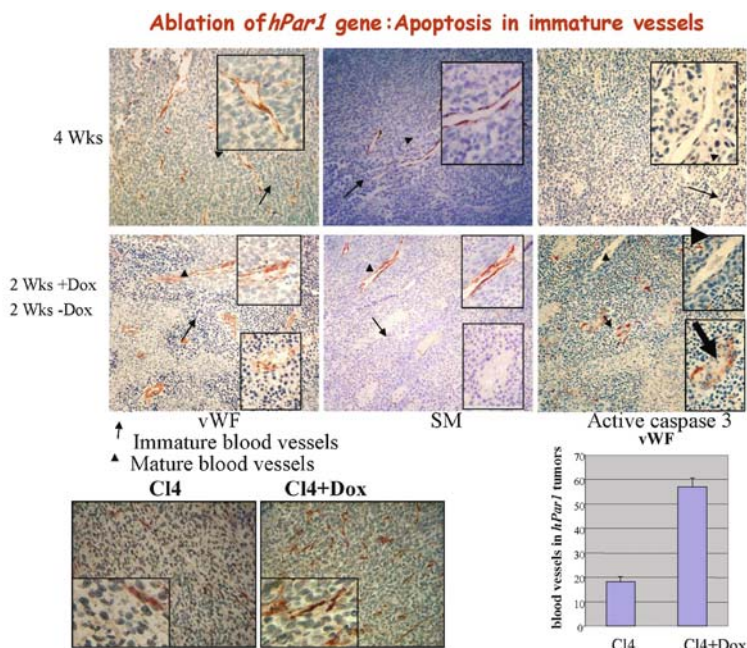


Fig. 10.3 Ablation of *hpar1* gene expression in a Tet-on *hPar1* inducible rat prostate carcinoma AT2.1 leads to apoptosis of immature blood vessels. (a) AT2.1/Tet-On/*Par1* C14 cells injected subcutaneously, and tumors were allowed to develop either for a period of 4 weeks (doxycycline (Dox) in the drinking water) or 2 weeks (doxycycline and withdrawal of doxycycline for an additional 2 weeks). Tumor sections (5 μ m) were processed and analyzed for levels of angiogenesis. Although total blood vessel network is detected following staining with vWF, staining with smooth muscle (SM) α -actin detects mature blood vessels (middle). Positive staining of active caspase-3, however, is seen only in immature blood vessels, and not in mature smooth muscle α -actin-positive vessels. Representative histograms showing the statistics of blood vessels in the different sections (ii). (b) Increased numbers of blood vessels are seen in sections grown in the presence of the *hPar1* gene (presence of doxycycline; C14 + Dox) compared with the section exhibiting no *hPar1* expression (C14). Right: histogram represents 58 vessels per slide in the presence of *hPar1* (C14 + Dox) compared with 18 vessels in the slide without *hPar1* expression (C14) (see Color Plates)

compared with the A375SM-derived tumor sections. The activation of PAR₁ induced pAkt/PKB, abrogated serum-deprived Bim_{EL} induction, and also markedly inhibited Bax levels. On the other hand, SiRNA silencing of *hPar1* induced the expression of Bim_{EL}, a direct substrate of Akt/PKB, and also induced the expression of active caspase-9 and caspase-3. Together, these results identify PAR₁ as a survival factor that protects the cells from undergoing apoptosis (Salah et al. 2007a). In parallel, in a tetracycline-inducible *hPar1* expression system of rat prostate cancer, we obtained an essentially similar outcome in which *hPar1* gene withdrawal resulted in blood-vessel-induced apoptosis. When tumors were allowed to develop for 2 weeks in the presence of the inducer, doxycycline, in the animals' drinking water, followed by the withdrawal of the *hPar1* gene by elimination of doxycycline for 2 additional weeks, and compared with tumor growth for 4 weeks (continuous presence of doxycycline), the following results were observed. When the blood vessel network was analyzed in xenografts of each group the ablated *hPar1* group consistently showed fewer, especially immature, blood vessels, whereas the mature vessels were essentially unaffected. No effect was noticed in mature blood vessels following *hPar1* shutdown. This, however, was not the case when immature blood vessels were analyzed. Following the withdrawal of the *hPar1* gene, active caspase-3 was observed in the reminiscent topographical location of an immature blood vessel, whereas none was seen in the mature blood vessel (Fig. 10.3). These data indicate that the silencing of *hPar1* initiates an apoptotic pathway in the immature sprouting blood vessels. This nonetheless markedly reduces the complexity of the blood vessel network and diminishes delivery of nutrients and other essential supplies to the center of a tumor forming an apoptotic center.

10.8 Transcriptional Regulation of Human *Par1*

Transcriptional regulation plays a central role in the molecular pathways underlying the preferential expression of gene network leading to cancer growth and metastasis. Deciphering the transcriptional pathways that regulate individual genes directly involved in cancer development is important for identifying essential determinants governing cancer initiation (Fearon and Vogelstein 1990; Liotta and Petricoin 2000). For example, analyses of the human *Par1* promoter revealed the presence of androgen response elements (ARE), which establish a functional androgen hormone regulation in prostate cancer advancement. Consistent with the hypothesis that androgen regulates *hPar1* expression in vivo, prostate cancer biopsy specimens showed high *hPar1* levels in tumor glandular cells, whereas tumor tissues taken from the same individual after hormone ablation treatment exhibited very little to no *hPar1* expression, as well as a markedly reduced tumor size (Salah et al. 2005). Using biochemical analyses of EMSA and ChIP (chromatin immunoprecipitation assays) we have demonstrated a functional androgen motif in the *hPar1* promoter based on physical binding in vivo between the androgen receptor and the gene promoter as well as *hPar1*-

promoter luciferase activities. However, a major drawback of hormone ablation therapy is that the reduction in tumor size is temporary; the tumor eventually proceeds to the hormone-resistant stage (Chen et al. 2004). It turned out that although we initially found androgen regulation of the *hPar1* gene (e.g., elicited by androgens and markedly reduced after hormone withdrawal therapy), high *hPar1* expression levels were, surprisingly, also found in the hormone-resistant prostate malignant carcinoma (Salah et al. 2005). This pointed out additional options for the regulation of *hPar1* overexpression in tumor malignancies. In addition, when a wide range of epithelial malignancy biopsy specimens were incubated with Dig-UTP-labeled *hPar1* riboprobes for in situ hybridization analyses, high *hPar1* RNA expression levels were obtained. Thus, at least in part, it is proposed that the *hPar1* overexpression is at the RNA level. Fluorescence in situ chromosome hybridization (FISH) for the determination of gene amplification on DNA copy numbers, performed either in high (e.g., CL1, prostate carcinoma) or low metastatic (e.g., LNCaP) cells, showed no difference in the number of gene copies, indicating that *hPar1* overexpression does not stem from gene amplification. We therefore analyzed transcription rate and mRNA stability to determine the mechanism underlying elevated *hPar1* transcripts. To evaluate *hPar1* mRNA stability, cells treated for various times with the transcription inhibitor agent DRB, followed by RNA isolation and northern blot analysis, revealed similar degradation rates regardless of whether high or low metastatic cancer cells were examined (Salah et al. 2007b). In contrast, when nuclear extracts from high and low metastatic cells were prepared for the evaluation of transcript elongation rates by run-on assay, we found markedly enhanced rates of *hPar1* expression in CL1 and PC3 aggressive prostate cancer cells relative to LNCaP, with low levels of *hPar1* expression (Salah et al. 2007b). We therefore concluded that enhanced levels of *hPar1* RNA in malignant cells are primarily due to increased transcription rates. Indeed, previous studies showed an inverse correlation between the expression of activator protein-2 α (AP-2 α) and PAR₁, as well as a direct correlation between Sp1 transcription factor and PAR₁ expression (Tellez et al. 2007). This suggests that increased PAR₁ expression is due to an altered ratio of AP-2/Sp1 (e.g., loss of AP-2 and elevated Sp1), with an increased proportion of Sp1 expression. This hypothesis was further verified by microarray assay of melanocyte tissues (Ruiz et al. 2001). To identify the transcription factors potentially involved in *hPar1* overexpression we searched for a candidate that may impinge on *hPar1* transcription, depending on the tumor. In prostate cancer, for example, we found a specific association with the early growth response-1 (*Egr-1*) gene. Analysis of the promoter genomic sequence revealed a potential *Egr-1* motif between -354 and -335 bps. Co-transfection of wild-type *Egr-1* expression vector and *hPar1* promoter-luciferase reporter (Luc-F1) into 293T cells showed a fourfold increase in luciferase activity as compared with luciferase activity in the absence of *Egr-1*.

Bombesin is a neuroendocrine peptide known to enhance *Egr-1* binding at the putative *Egr-1*/specific protein 1 (Sp1) binding motif (Xiao et al. 2005). Treatment of DU-145 cells with bombesin increased the levels of *hPar1* expression in a dose- and

time-dependent manner. Bioinformatic analysis predicted the presence of multiple *Egr-1* binding sites located between -354 and -335 bps in the 5'-UTR of *hPar1*. The designated region offers five combinatorial options to generate the *Egr-1* motif. EMSA analysis shows that induction of EGR-1 often displaces Sp1, Sp3, and Wilm's tumor 1 (Adamson et al. 2003). Specific binding was judged based on increased band intensity after bombesin treatment, effective competition in the presence of excess cold oligonucleotides, and no effect on the binding properties in the presence of mutant *Egr-1* oligos. In addition, in the presence of specific EGR-1 antibodies, a classical super-shift is obtained (Salah et al. 2007b).

ChIP analysis was performed on chromatin fragments immunoprecipitated from cultured DU-145 prostate cancer cells before and after bombesin treatment using either anti-EGR-1 antibodies or a control IgG. The DNA from the immunoprecipitated complex was then extracted and amplified using an appropriate set of primers to cover the region between -402 and -131 bp, which includes the proposed motif between -354 and -335 bp. A fourfold increase in PCR product was observed from cells treated with bombesin compared with untreated cells. When control IgG was used to immunoprecipitate chromatin, only minimal amounts of PCR products were observed as a nonspecific background.

The functional relevance of *Egr-1*-induced *hPar1* expression was demonstrated in vitro by marked Matrigel invasion using Boyden chambers. When *hPar1*-SiRNA-infected cells were applied to the same Matrigel invasion assay before and after *hPar1*-silencing expression, a marked reduction in invasion was obtained, parallel to reduced expression levels. In contrast, a significant increase in Matrigel invasion was observed in the presence of bombesin treatment.

When prostate tissue biopsy specimens were immunostained for the relative distribution and localization of either PAR₁ or EGR-1, no staining was observed in normal-appearing tissue structures, while a strong and positive staining was seen in neoplastic tissues.

We also evaluated the interrelationship between p53, a tumor suppressor gene, and *hPar1* levels of expression. The p53 gene is the most frequent target of genetic alteration yet identified in human cancers, affecting more than 50% of all tumors (Joerger and Fersht 2007; Soussi 2007). p53 encodes a nuclear transcription factor that accumulates in the cell in response to a variety of stress conditions. Under stresses like hypoxia, DNA damage, viral infection, heat shock, and oncogenic activation, p53 induces either growth arrest or apoptosis, depending on the severity of the stress-induced damage. In unstressed cells, p53 has a short half-life, and is thus maintained at low levels. p53 binds specifically to a double-stranded target DNA, composed of two decameric "half-site" motifs separated by up to 13 bp (el-Deiry et al. 1992), to induce target gene transactivation. Under stress conditions, when p53 is expressed in a wild-type form, it represses a large number of downstream genes. In tumors, however, the delicate balance between oncogenes and tumor suppressor genes is interrupted, as portrayed via the disappearance of p53 or its presence in altered mutated forms. In most cases, the mutation is located in the DNA-binding core domain of the protein (Olivier et al. 2002). The functional consequences of p53 cancer mutations are complex (Blagosklonny

2000; Sigal and Rotter 2000) and several systematic studies have shown that while certain mutants result in a complete loss of p53 function, others exhibit altered transactivation spectra or retain function at lower temperatures (Kato et al. 2003; Resnick and Inga 2003; Menendez et al. 2006; Dearth et al. 2007). We observed that *hPar1* expression is low in cancer cell lines expressing wild-type p53 (e.g., LNCaP and MCF7), while it is overexpressed in cell lines lacking p53 expression (e.g., PC3 and CL1), or expressing mutant (*mt*) forms (e.g., DU145 and HT29) of p53. Thus, we propose that p53 may serve as a potential regulator for the fine tuning of *hPar1* expression during cancer development. To further demonstrate a direct correlation between p53 and *hPar1*, we used the H1299 lung adenocarcinoma cells that are p53-negative or stably transfected with a temperature-sensitive (*ts*) Val135 mutant form of p53 (H1299Val135 cells). This mutant protein contains a substitution of cysteine to valine at position 135, and possesses wild-type activity at 32°C, and a mutant, inactive conformation at 37°C. We tested *hPar1* mRNA expression in temperature-sensitive (*ts*) cells in which a temperature shift from 37 to 32°C results in downregulation of *hPar1* mRNA (Salah et al. 2008). To show decisively that the correlation obtained with *hPar1* levels is a result of p53 effect, and is not due to another genetic change acquired by these cells, we made use of p53 SiRNA stably expressing MCF7 cells. Detectable *hPar1* levels are seen in these cells upon p53 loss, as compared with nondetectable *hPar1* levels in the parental cells which express high p53 levels. In addition, when *mt* p53 expression is lost, *hPar1* expression is similarly downregulated. These data clearly suggest a reciprocal correlation between wild-type p53 and *hPar1* expression, and a proportional direct correlation between *mt* p53 and *hPar1* levels. To evaluate the effect of p53 on *hPar1* functional activity, we tested whether PAR₁ signaling pathway is negatively affected. For this purpose, we examined the phosphorylation status of a known PAR₁ target protein, focal adhesion kinase (FAK). FAK has been shown to rapidly undergo phosphorylation upon PAR₁ activation. When we incubated H1299Val135 cells at either 32 or 37°C, and then analyzed p-FAK levels following activation with SFLLRN, PAR₁ ligand peptide, the following data were obtained. While nearly no change in phosphorylated FAK levels was observed in cells incubated at 32°C (wild-type p53 is expressed), a marked increase was observed in cells incubated at 37°C, at which *mt* p53 is expressed. To further show that PAR₁ functionality as well as *hPar1* expression is attenuated, we performed a Matrigel invasion assay using H1299Val135 cells. A marked inhibition in cell invasion capability was observed upon a temperature shift from 37 to 32°C, indicative of the direct impact of wild-type p53 vs. inactive mutant p53 on PAR₁ function. To demonstrate that this phenomenon correlates with attenuated *hPar1* gene expression and not to other p53 target genes, we re-expressed *hPar1* ectopically using retroviral infection. Indeed, *hPar1* expression was observed by RT-PCR after infection, demonstrating that the induction in *hPar1* expression is not affected by a temperature shift. When we performed a Matrigel assay with these cells (expressing ectopic *hPar1*), we observed loss of the temperature shift effect on cell invasion capability, clearly suggesting that re-expression of *hPar1* rescued p53-induced invasion inhibition (Salah et al. 2008).

10.9 Concluding Remarks

While analyzing levels of *hPar1* expression in epithelial malignancies we observed a direct correlation between high *hPar1* expression levels and tumor metastasis. This holds true both in the physiological invasion process of placenta implantation to the uterus deciduas during the first trimester of pregnancy and in the pathological malignant invasion process. In a transgenic mouse model targeted to overexpress *hPar1* in the mammary glands, whole mounts from transgenic mice showed markedly increased branching and a higher complexity of alveoli proliferation compared with age-matched wild-type littermates. The pronounced hyperplasia is characterized by the strikingly stabilized levels of β -catenin, as well as *wnt-4* and *-7b*. This novel association between *hPar1* and the oncogenic β -catenin remains to be explored and is a subject of our current studies. Identification of such linkers may lead to the development of therapeutic medicaments for cancer. While *hPar1* behaves as a prosurvival and proangiogenic gene, the molecular machinery underlying *hPar1* overexpression is poorly addressed. We provide evidence showing that *hPar1* overexpression stems mainly from an increased transcriptional rate on a background of equal stability. We have identified *Egr-1* as a major transcription factor, eliciting *hPar1* gene expression in prostate cancer progression on the one hand, and p53 as a tumor or suppressor gene regulating levels of *hPar1* on the other. It still remains to determine the individual transcription factors and/or tumor suppressors that are critically involved in the regulation of *hPar1* transcription activity in the context of tumor progression.

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Chapter 11

Anti-thrombotic Therapy in Cancer Patients

Gloria A. Petralia and Ajay K. Kakkar

Abstract A complex relationship exists between the coagulation system and tumour cells, with common mechanisms linking haemostasis and malignancy. Venous thromboembolism (VTE) is the second most common cause of death in cancer patients and it is estimated that about 1 in 7 patients die of avoidable pulmonary embolism (PE), rather than the cancer itself. Treating a patient with cancer requires a multidisciplinary approach, whether the intention is to cure or to palliate; life expectancy may be improved in certain patients by aggressive intervention, but if a limited life span is expected, preserving quality of life becomes paramount. Cancer is an independent risk factor for VTE, but cancer patients are also at higher risk of bleeding complications and recurrence.

Exciting data from prospective randomised clinical trials in cancer patients have now established that low molecular weight heparins (LMWHs) are the agents of choice both in the primary prevention of venous thromboembolic disease in cancer patients undergoing surgical intervention and in the treatment and long-term secondary prevention of recurrent VTE in cancer patients who develop a thrombosis. These agents can be given safely without need, in general, for routine laboratory monitoring in cancer patients.

Anti-thrombotic drugs, and in particular LMWHs, have recently been demonstrated to have potential anti-tumour effects. The survival advantage associated with LMWH usage may be due to a combination of (1) prevention of fatal thromboembolic disease; (2) interference with the coagulation proteases that influence tumour phenotype and (3) a potential direct anti-tumour cell effect of heparin itself.

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11.1 Introduction

Anti-thrombotic agents are classically used for the prevention and treatment of venous and arterial thromboembolic disease. They have more recently been evaluated for anti-cancer properties (Petralia et al. 2005).

In order to explore the role of anticoagulants in cancer patients we will need to understand the two-way relationship between malignancy and thrombosis and take into consideration the challenges inherent to the treatment of cancer patients. We will review epidemiological and experimental data to support prophylactic and therapeutical regimes as suggested by current guidelines. Finally, we will collate evidence supporting that the use of anti-thrombotic agents is linked to a benefit in survival that cannot be explained exclusively by their prevention or treatment of thromboembolic events.

Pathological thrombus formation is promoted by a triad of factors first described Virchow in 1856 (Virchow 1856): venous stasis, vascular trauma and increased blood coagulability. In cancer patients the complex interplay involving tumour-, patient- and therapy-related factors influences the genesis of Virchow's triad (Table 11.1).

The delicate balance between the coagulation and fibrinolytic systems ensures a prompt and regulated response to vascular injury; this facilitates haemostatic plug formation, with subsequent repair and remodelling after the acute injury has been stabilised; all the time avoiding pathological intravascular occlusive thrombosis. In the hypercoagulable state found in malignancy this balance is disturbed, the reasons behind that are varied and multifactorial.

The capacity of tumour cells to express procoagulant molecules such as tissue factor (TF) (Gordon 1992; Kakkar et al. 1995a, b) and cancer procoagulant (CP) (Letai and Kuter 1999; Lee 2002) may allow them to influence thrombosis directly. They release inflammatory mediators such as tumour necrosis factor (TNF) and interleukin proteins (such as IL-1) allowing them to promote coagulation indirectly (Prandoni 1997), or by stimulating endothelial and mononuclear cells to secrete procoagulant molecules and by playing a role in platelet activation (Lee 2002).

Table 11.1 Virchow's triad in malignancy

Venous stasis: <i>Alteration in blood flow</i>	Thrombocytosis increases blood viscosity
	Extrinsic compression by tumour growth or invasion can cause mechanical blockage
	Patient immobility due to cancer complication or therapy
Vascular trauma: <i>alteration in blood vessels</i>	Angiogenic stimuli can create a web of aberrant vessels that cause turbulent flow
	Direct invasion by a tumour leads to mechanic endothelial trauma
	Intravenous administration of chemotherapeutic agents can cause endothelial irritation
Blood hypercoagulability: <i>alteration in blood components</i>	Dysfunctional endothelium can lose its antithrombotic properties
	Increase in procoagulant activities
	Decrease in anticoagulant activities
	Increase in overall platelet activity

Cancer procoagulant is a calcium-dependant cysteine protease, that has been found to be expressed by a variety of tumours (Donati, et al. 1986; Tallman and Kwaan 1992), having the capacity to directly activate factor X independently from the presence of TF/VIIa complex (Letai and Kuter 1999).

Tissue factor is physiological initiator of coagulation leading to thrombin production. Being expressed by endothelial cells, it is usually not exposed to circulating blood until there is a disruption in the endothelium continuity or vascular damage. TF then interacts with the circulating factors VII and VIIa to form the TF/VIIa complex. On TF/VIIa complex formation the intracellular protein filamin A, which is implicated in cell motility, is recruited to the cytoplasmic tail of TF (Ott et al. 1998). The TF/VIIa complex (Rickles et al. 2001) and tumour hypoxia (Shweiki et al. 1992) also up-regulate the expression of VEGF (Poon et al. 2001). Its ability to promote megakaryocyte maturation may explain increased platelet turnover in cancer patients (Mohle et al. 1997) and cancer-related thrombocythemia (Edwards et al. 1987). Systemic activation of blood coagulation in cancer patients appears to be TF dependent with resulting activation of the extrinsic and common pathways of blood coagulation. TF expression in tumour cells is associated with down-regulation of thrombospondin, an anti-angiogenic factor (Zhang et al. 1994). Circulating endothelial cells do not express TF, but do so if stimulated and may, therefore, have a role in intratumoural coagulation and angiogenesis (Beerepoot et al. 2004)

Various tumour cell lines have expressed TF: sarcoma, melanoma, neuroblastoma, lymphoma and acute promyelocytic leukaemia (Rickles et al. 1995). TF expressed by epithelial-derived tumours, such as the ductal epithelial elements of pancreatic adenocarcinoma, results in transformation from a benign to malignant phenotype; with its expression correlating with histological grade (Kakkar et al. 1995a). TF over-expression, using techniques of gene transfer in an immunodeficient mouse model of pancreatic carcinoma is associated with enhanced primary tumour growth in vivo (Kakkar et al. 1999).

A similar effect has been elicited in the sarcoma model where experimental over-expression of TF leads to tumour growth enhancement (Zhang et al. 1994). This TF manipulation was associated with a corresponding increase in tumour production of vascular endothelial growth factor (VEGF), which promotes TF expression in adjacent endothelial cells; and decrease in the anti-angiogenic regulatory protein thrombospondin.

In breast cancer, TF expression has been linked to invasion (Contrino et al. 1996), whilst in human hepatocellular carcinoma high levels of TF are associated with poor prognosis and a predictor of poor survival (Poon et al. 2003).

When TF expression was knocked down in human metastatic melanoma cells, 44 known human genes were significantly up-regulated and 228 genes significantly down-regulated (Wang et al. 2004). These had potential effect on a variety of cellular pathways including transcription, translation, cell communication, and cell growth/death. Interestingly these gene expression changes were associated with a reduction in pulmonary metastasis.

TF plays an essential role in embryonic vasculogenesis (Carmeliet et al. 1996), and appears to have an important role in controlling pathological angiogenesis. The cytoplasmic tail of TF has a regulatory function in protease activated receptor 2 (PAR₂)-dependent angiogenesis, resulting in uncontrolled PAR₂-dependent vascular growth (Belting et al. 2004).

Thrombin/PAR signalling results in up-regulation of TF expression, and increased invasiveness of breast (Levine et al. 2003) and pancreatic (Taniguchi et al. 1998; Kakkar et al. 1998; Wojtukiewicz et al. 2001) cancer lines.

11.2 Clinical Challenges and Epidemiology

VTE may be the first clinical manifestation of undiagnosed malignancy (Prandoni 1997; Kakkar and Williamson 1999; Di Carlo et al. 1999). Idiopathic VTE is associated with an increased risk of developing cancer of between 1.3- and 3.2-fold, when compared to the native population (Sorensen et al. 1998; Baron et al. 1998). Conversely, patients with an established diagnosis of malignant disease are at increased risk of VTE. A relationship between thromboembolic disease and cancer was first identified in 1865 by Armand Trousseau in his renowned paper on thrombophlebitis migrans (Trousseau 1872).

The clinical manifestations of venous thromboembolism (VTE) vary within a wide range that spans from asymptomatic deep vein thrombosis (DVT) to fatal pulmonary embolism (PE). It is estimated that 10–20% of patients dying with cancer may be dying of underlying, potentially preventable PE, rather than the cancer itself (Shen and Pollak 1980). VTE is the second most common cause of death (Rickles and Edwards 1983) in patients with malignant disease. Treatment of VTE is estimated accounts for 6% of inpatient bed usage on medical oncology wards, with up to 15% of cancer patients experiencing a symptomatic thromboembolic event throughout their cancer history (Harrington et al. 1997).

It appears that tumour histology is related to the risk of developing PE. In a necropsy study (Svendsen and Karwinski 1989), ovarian cancer had the highest rates (34.6%), followed by malignancies of the extrahepatic biliary system (31.7%) and of the stomach (15.2%); whereas the lowest rates (0.5–6%) were in cancer of the oesophagus and larynx, myelomatosis and lymphoma.

Cancer is a recognised independent predictor for VTE and patients with cancer undergoing surgery are at increased risk of developing post-operative DVT (41% compared with 26%; RR 1.96; $P = 0.04$) (Kakkar et al. 1970) or PE (1.6% vs. 0.4%; $P < 0.05$) (Rahr and Sorensen 1992) when compared to the non-cancer population. Rates of VTE in studies that have reported outcome for cancer and non-cancer subjects separately are detailed in Table 11.2.

Symptomatic post-operative VTE following cancer surgery have been reported as 2.83% in general surgery, 2.0% in gynecological surgery, and 0.87% in urological surgery with 40% of the events occurring after the third post-operative week (Agnelli et al. 2006). Interestingly VTE was the lead cause of death at day-30 and

Table 11.2 Incidence of post-operative VTE: cancer vs. non-cancer patients

	Cancer (%)	Non-cancer (%)
Kakkar et al. (1970)	24/59 (41%)	38/144 (26%)
Hills et al. (1972)	8/16 (50%)	7/34 (21%)
Walsh et al. (1974)	16/45 (36%)	22/217 (10%)
Rosenberg et al. (1975)	28/66 (42%)	29/128 (23%)
Rem et al. (1975)	16/30 (53%)	16/65 (25%)
Gallus et al. (1976)	17/76 (22%)	49/306 (16%)
Allan et al. (1983)	31/100 (31%)	21/100 (21%)
The_Multicenter_Trial_Committee (1984)	62/304 (20%)	113/707 (16%)
Kakkar and Murray (1985)	21/310 (6.8%)	10/597 (1.7%)
Sue-Ling et al. (1986)	12/23 (52%)	16/62 (26%)
Kakkar et al. (1993)	25/1,407 (1.8%)	16/2,402 (0.7%)
Total	260/2,436 (10.7%)	337/4,762 (7.1%)

was responsible for 46.3% of deaths (overall death rate 1.72%) (Agnelli et al. 2006).

Cancer is an independent risk factor for VTE despite thromboprophylaxis and cancer patients who experience a thromboembolic episode are at a higher risk for the development of subsequent recurrent thrombotic episodes (Gallus 1997; Kakkar and Murray 1985; Huber et al. 1992; Bergqvist et al. 1995). The rate of recurrent VTE after initial treatment with longer term anticoagulation were 6.8% (95% CI, 3.9–9.7%) for 661 non-cancer and 20.7% (95% CI, 15.6–25.8%) for 181 cancer patients, respectively, in one study (Prandoni et al. 2002). Further studies have supported these findings: 9.0% for 1,039 noncancer patients vs. 27.1% in 264 cancer patients ($p=0.003$) in one study (Hutten et al. 2000) and 20.8% vs. 6.5% (Hazard ratio (HR) 3.2, 95% CI, 1.9–5.4) in another (Prandoni 2002).

The prothrombotic effect of certain chemotherapeutic agents is documented and may in part be mediated by damage to the endothelial cells (Boraks et al. 1998). For example, in patients receiving breast cancer treatment the reported incidence of VTE ranges from 1.7 to 17.6% (Petrulia and Kakkar 2004). The risk in those patients seems to be cumulative with increase of VTE risk when combination chemotherapy is administered compared to single agent (Levine et al. 1988). Post-operative adjuvant chemotherapy in post-menopausal patients with early stage (I and II) breast cancer seems also to be responsible for an increase of VTE rates from 0.7 to 2.3% ($p=0.001$) (Clahsen et al. 1994; Saphner et al. 1991).

Endocrine therapy in breast cancer, for example in the use of Tamoxifen, increases DVT risk in both pre-menopausal (2.3% vs. 0.8%, $p=0.003$) and post-menopausal (8.0% vs 2.3%, $p=0.003$) women (Saphner et al. 1991). Its use in combination with chemotherapy increased DVT risk when compared to tamoxifen alone in a group of stage II breast cancer from 1.4 to 9.6% ($p=0.0001$).

Radiotherapy further contributes to the thromboembolic risk. In patients with rectal cancer receiving neo-adjuvant radiotherapy, an increased VTE rate was reported during the first 30 days following the planned surgery (Goldberg et al. 1994) in those patients that underwent chemotherapy compared to those that did

not receive the adjuvant treatment. Those results were confirmed in a later 5-year follow up study: 7.5% for radiotherapy vs. 3.6% for no radiotherapy ($p = 0.001$) (Holm et al. 1996).

Unfortunately cancer is also a risk factor for anticoagulant-related bleeding: the incidence of clinically important anticoagulant-related bleeding was reported as 4.9% (95% CI, 2.5–7.4%) in 661 non-cancer vs. 12.4% (95% CI, 6.5–18.2%) in 181 cancer patients (Prandoni et al. 2002). Again this data is further supported with published rates of 13% vs. 2%, respectively ($p = 0.002$) (Hutten et al. 2000); 12.4% vs. 4.9% (HR 2.2, 95% CI, 1.2–4.1) (Prandoni 2002) and 16.1% vs. 7.4% (Kakkar et al. 1993).

11.3 Thromboprophylaxis

It has emerged that methods of prophylaxis for VTE in cancer patients currently show marked regional variations, are rarely employed in medical oncological patients whilst surgical patients may not receive recommended thromboprophylaxis due to a fear of bleeding complications (Wolff 2003).

Broadly speaking preventative methods can be classified in mechanical and pharmacological. Amongst the mechanical only the use of graduated static compression stockings, usually in combination with pharmacological methods of prophylaxis, have been proven to be effective in the cancer population. They are commonly used in post-operative surgical patients in such fashion and reduce the incidence of post-operative DVT (Allan et al. 1983). Inferior vena cava filters have been primarily evaluated in the setting of established VTE disease, with their placement to prevent PE (Decousus et al. 1998); they do not appear as effective in protecting against fatal PE in cancer patients (Athanasoulis et al. 2000; Millward et al. 1994).

Oral anticoagulants (OAC), such as warfarin, act as vitamin K antagonists, preventing post-translational carboxylation of clotting factors II, VII, IX and X in the liver. Regular monitoring of the anticoagulant activity is required using the international normalised ratio (INR) for consequent dose adjustment; they interact with a variety of other pharmaceutical agents and are adversely effected by patient's unfavourable nutritional status. Achieving therapeutic anticoagulation with OAC is more difficult in cancer patients than in non-cancer patients (56.9% of the time vs. 43.3%; $P < 0.0001$) (Bona et al. 1995) The difficulty in achieving a safe and therapeutic INR can also be problematic in preventing post-operative VTE (Harrison et al. 1997).

Unfractionated heparin (UFH) has a pentasaccharide sequence that binds to the endogenous anticoagulant protein antithrombin (AT) enhancing its ability to inhibit both thrombin and factor Xa. Low dose UFH is given subcutaneously for VTE prophylaxis. The use of UFH may be complicated by the development of heparin-induced thrombocytopenia (HIT) (Hirsh et al. 1998; Brill-Edwards et al. 1993; Hull et al. 1997). The effect of UFH may be rapidly reversed with protamine sulphate (Kim et al. 2004).

Low molecular weight heparin (LMWH) is prepared by chemical or enzymatic degradation of UFH. LMWH has a lower average molecular weight than UFH, allowing effective absorption from the subcutaneous tissue. Its mechanism of action is similar to UFH, but with diminished inhibition of thrombin. Its affinity for plasma proteins, platelets, macrophages and endothelium is reduced, increasing the predictability of its anticoagulant response, with a longer plasma half-life (3.5–4.5 h) and increased bioavailability (>85%). Subcutaneous administration is therefore facilitated on a once daily basis allowing for outpatient use. In addition, LMWH has a lower incidence of HIT (Warkentin et al. 1995; Hirsh et al. 1998), lower risk of bleeding (Siragusa et al. 1996; Lensing et al. 1995; Leizorovicz et al. 1994; Dolovich et al. 2000; Gould et al. 1999), and has not been associated with osteoporosis (Kakkar and Williamson 1997; Muir et al. 1997; Shaughnessy, et al. 1995; Monreal et al. 1994).

11.3.1 Primary Surgical Thromboprophylaxis

Low dose UFH is commonly administered subcutaneously at a dose of 5,000 units, starting 2 h prior to surgery, and continued two or three times a day. There is early evidence (Rem et al. 1975; Gallus et al. 1976) to support the use of post-operative heparin in cancer surgery. A large meta-analysis (Clagett and Reisch 1988) evaluated 29 trials in which surgical patients received UFH and evaluated 919 cancer patients finding a significant reduction in the incidence of VTE from 30.6 to 13.6% in patients receiving UFH ($p < 0.001$). UFH was also shown to reduce mortality due to PE from 1.6 to 0.4% in one randomised trial (Anonymous 1975).

The use of LMWH has now been extensively proven to be at least as safe and effective, if not more effective, than UFH (Petrulia and Kakkar 2004). In a study comparing once daily LMWH vs. UFH three times a day for elective abdominal curative surgery for cancer total VTE rates were 16.5% (104/631), UFH rates were 18.2% and LMWH rates were 14.7% with no difference in bleeding morbidity or mortality (Anonymous 1997). In a systematic review of 7,639 patients pooling data from 26 randomised controlled trials of surgical oncology patients DVT rate without prophylaxis were 35.2%, reduced to 12.7% with heparin (UFH or LMWH) and further decreased to 5% with the combination of heparin and mechanical prophylaxis (Leonardi et al. 2007).

With higher doses of LMWH (5,000 units vs. 2,500 1995 units) in 2,097 2007 surgical cancer patients, VTE rates were improved at the higher dose from 14.9 to 8.5% ($P = 0.001$) with no increase in bleeding rates (Bergqvist et al. 1995). A systematic review found similar results, with significantly reduced rates of DVT apparent with higher dose LMWH and UFH (8% high vs. 13.4% low $P < 0.0132$) in pooled analyses from 17 randomised controlled trials (Leonardi et al. 2007).

Longer prophylaxis with LWMH for up to 4 weeks after abdominal or pelvic surgical procedures for cancer reduces VTE rates at 4 weeks from 12 to 4.8.0% ($P = 0.02$) and at 3 months from 13.8 to 5.5% ($P = 0.01$) (Bergqvist et al. 2002).

LMWH has also been shown to be safe and effective in neurosurgery, despite the risk of intracranial bleeding showing a 48% VTE risk reduction with no increase in bleeding (Iorio and Agnelli 2000).

11.3.2 Prevention of VTE in Non-Surgical Patients

Prophylactic levels of OAC are achieved by adjusting the dose to maintain an International normalised ratio (INR) of between 2.0 and 3.0. In 311 patients undergoing chemotherapy for stage IV breast cancer randomised to very low dose warfarin (INR 1.3–1.9) and placebo, there was a risk reduction in VTE (0.6 vs. 4.4%; $P = 0.031$) with no significant increase in bleeding rates. (Levine et al. 1994).

Incidence of VTE was significantly reduced in the group receiving warfarin (9 vs. 37%; $P = 0.001$) amongst 84 cancer patients with indwelling central catheters randomised to warfarin (1 mg) or placebo (Bern et al. 1990); similar results have been obtained with LMWH given in a dose of 2,500 1996 units daily where rates were reduced from 60 to 6% with this therapy (Monreal et al. 1996).

11.4 Treatment of Venous Thromboembolism

Recommendation for the treatment of VTE in the cancer setting are similar those for the general population-initial treatment with either intravenous UFH or subcutaneous LMWH for at least 5–7 days. DVT treatment with intravenous UFH starts with a dose of 5000 Units followed by continuous infusion to maintain an activated partial thromboplastin time (APTT) of 1.5–2.0 times the control value (Petralia and Kakkar 2004; Buller et al. 2004). LMWH is given in a twice-a-day or daily regime that does not require dose monitoring offering the opportunity to treat the majority of uncomplicated DVT cases safely, effectively, and cost-effectively in an outpatient environment. In addition, LMH is not only as effective as UFH in the initial treatment as assessed by the prevention of recurrent VTE (odds ratio 0.85) but is associated with a significant reduction in the risk of bleeding complications (odds ratio 0.57; $P = 0.05$) (Gould et al. 1999).

11.5 Prevention of Secondary Recurrence

Following initial treatment with heparin, long-term secondary thromboprophylaxis is usually provided with Vitamin K antagonists. Unfortunately in cancer patients it is more difficult to maintain a therapeutic INR (Bona et al. 1995); there may be either the need to interrupt OAC for thrombocytopenia secondary to disease of therapy, or the need for interventional procedures for management of the cancer.

LMWHs have potential advantages in that they can be provided in a fixed daily dose, in general they do not require laboratory monitoring, and their anticoagulant effect can be easily interrupted by omitting a single dose of therapy.

The CLOT study (Lee et al. 2003) compared long-term OAC vs. LMWH showing a 52% VTE reduction (from 17 to 9%; $P = 0.002$) at 6 months in favour of the LMWH group of cancer. The pilot study ONCENOX (Deitcher et al. 2003) randomised 122 patients with cancer-associated thrombosis to LMWH for 175 days or OAC therapy also for 175 days, showing no significant differences between the two groups.

11.6 Anti-Thrombotic and Cancer Survival

The association of cancer and VTE seems to be associated with poorer outcomes; be that because it is commoner in more aggressive tumours, it is less manageable in the cancer patients or is simply related to the activation of the coagulation cascade and its influence of the tumour biology is still to be determined (Lee et al. 2003). Nonetheless, 6-month death probability goes from 15% in patients with cancer to 80% in patients with both the diagnosis of cancer and VTE (Kakkar 2003).

Patients with small cell lung cancer (SCLC) receiving warfarin showed a benefit in terms of survival (Sack and Bell 1977), whilst the addition of UFH to chemotherapy increased median survival from 261 to 317 days ($p = 0.001$) (Zacharski et al. 1984). Meta-analyses of retrospective data on cancer patients receiving DVT treatment suggest an improved 3-month survival of about 10–20% for such patients (Lebeau et al. 1994).

The Fragmin Advanced Malignancy Outcome Study (FAMOUS) trial (Gould et al. 1999) randomised 385 cancer patients to LMWH or placebo showing a 5% absolute increase in favour of the treatment arm (41% placebo, 46% dalteparin). A post hoc analysis of patients with good prognosis, revealed an increase in median survival from 24 months with placebo to approximately 43 months with dalteparin.

The CLOT comparison of LMWH vs. OAC showed no overall benefit at 1 year, but a subgroup of patients without metastasis at randomisation, survival rates were in favour of the LMWH group (80% vs. 64%) (Kakkar et al. 2004). In the MALT study (Lee et al. 2005), 302 cancer patients were randomized to LMWH or placebo showed a significant survival advantage at 12-month in favour of the treatment arm (39% vs. 27%; HR 0–75, $P = 0.02$).

The survival advantage associated with LMWH usage may be due to a combination of (1) prevention of fatal thromboembolic disease; (2) interference with the coagulation proteases that influence tumour phenotype and (3) a potential direct anti-tumour cell effect of heparin itself.

Direct anti-tumour effects of heparin, independent from its anti-thrombotic properties include (Klerk et al. 2003): anti-angiogenesis, inhibition of heparanase (Smorenburg and Van Noorden 2001), interference with P-selectin-mediated adhesion, apoptosis induction and modification of oncogene expression.

In an experimental model, heparin treatment attenuated metastasis formation by inhibiting P-selectin-mediated aggregation of tumour cell with platelets via cell-surface mucin ligands (Vlodavsky et al. 1999). Low anticoagulant activity heparins can inhibit lung colonisation in the Lewis lung carcinoma model (Borsig et al. 2001). UFH is able to bind platelet integrin $\alpha\text{IIb}\beta 3$ thus enhancing ligand binding and differentially modulating adhesion of cancer cells to vitronectin; a process potentially interfering with tumour cells invasion and metastasis. LMWH and chondroitin sulphate also induce a significantly reduced enhancement of this adhesion in a way that is dependent on the integrin β -chain (Da Silva et al. 2003; Yoshitomi et al. 2004).

Nasir et al. (2003) have demonstrated that administration of LMWH to tumour bearing mice in the NDST-2 knockout model where cells are unable to synthesize endogenous heparin, was able to induce tumour apoptosis.

11.7 Conclusion

A complex relationship exists between the coagulation system and tumour cells, with common mechanisms linking haemostasis and malignancy.

Exciting data from prospective randomised clinical trials in cancer patients have now established that LMWHs are the agents of choice both in the primary prevention of venous thromboembolic disease in cancer patients undergoing surgical intervention and in the treatment and long-term secondary prevention of recurrent VTE in cancer patients who develop a thrombosis. These agents can be given safely without need, in general, for routine laboratory monitoring in cancer patients.

Anti-thrombotic drugs, and in particular LMWHs, have recently been demonstrated to have potential anti-tumour effects. Although data from contemporary trials remain only partially convincing, further evaluation is now warranted to determine if coagulation modulation prolongs survival in cancer patients.

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Chapter 12

Thrombin Receptor Modulators: Medicinal Chemistry, Biological Evaluation, and Clinical Application

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Abstract The serine protease α -thrombin maintains haemostasis through its coagulant, anticoagulant, and platelet-activator functions. This enzyme also has important cellular effects involving cell proliferation, cytokine and growth factor release, and tissue remodeling, which are mediated by G-protein coupled receptors known as protease-activated receptors (PARs). Thrombin can activate three of the four PAR family members, and PAR₁ is the primary thrombin-responsive receptor in human cells. PAR₁ plays an important role during the response to tissue injury and the associated inflammatory processes. The blockade of PAR₁ offers a new approach for treating various disorders that depend on thrombin generation, including thrombosis and restenosis. Antagonists of PAR₁ will interrupt thrombin's receptor function, but not thrombin's proteolytic activity, thereby providing an alternative means to attenuate the pathological effects of thrombin. This chapter deals with the topic of PAR₁, with the key medicinal chemistry, pharmacology, and clinical aspects of PAR₁ antagonists, and with the topic of PAR₄. The full potential of PAR₁ antagonists has yet to be realized commercially, but the promise of novel therapeutics is reflected by two antiplatelet PAR₁ antagonists in advanced human clinical trials.

12.1 Introduction

The serine protease thrombin (EC 3.4.21.5) is generated in the circulatory system during activation of the blood coagulation cascade. This enzyme is central to the maintenance of haemostatic balance through its coagulant and anticoagulant properties (Hall et al. 1997; Leung and Gibbs 1997). In addition to its central role in haemostasis, thrombin mediates a host of cellular responses, that involve platelets,

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endothelial cells, smooth muscle cells, fibroblasts, inflammatory cells, neurons, and tumor cells. These cellular effects derive from thrombin's activation of specific cell-surface receptors, which are unique members of the G-protein-coupled receptor (GPCR) superfamily. Notably, these receptors are activated by proteolytic cleavage of their extensive N-terminal extracellular domain and are thus known as "proteinase-activated receptors" (PARs) (Derian et al. 2003b; Coughlin 2005; Traynelis and Trejo 2007). PAR₁, PAR₃, and PAR₄ are the PARs of relevance to thrombin-based biological responses, and their activation leads to GPCR-type signal transduction. By contrast, PAR₂ is not activated by thrombin; rather, it is activated by other serine proteases such as trypsin or tryptase.

Thrombin's cellular actions contribute to many pathological conditions, such as thrombosis, restenosis, atherosclerosis, neurodegenerative disorders (e.g., Alzheimer's disease), and inflammation (Barry et al. 2006; Chackalamannil 2006; Leger et al. 2006a); Maryanoff 2006; Luo et al. 2007; Meadows and Bhatt 2007; Sokolova and Reiser 2007). One of its most profound functions entails the activation and aggregation of blood platelets. Under normal physiological conditions, platelets play a key role in bleeding prevention via essential clot formation. However, platelets also exhibit a "dark side" under certain pathophysiological conditions, wherein they are central to arterial thrombosis and atherothrombosis. Uncontrolled thrombosis and/or atherothrombosis are the principal causes of the clinical symptoms of cardiovascular disease (CVD), such as acute coronary syndrome (ACS), myocardial infarction (MI), and ischemic stroke. Normally, platelets migrate to a site of tissue injury and seal the damaged blood vessel, first forming a single layer of cells, and then recruiting additional platelets into a platelet aggregate, or plug. In the case of atherothrombosis, platelet adhesion occurs shortly after an atherosclerotic plaque has ruptured, eroded, or become disrupted. The platelets are then activated mainly by exposed collagen fibrils and locally produced thrombin to generate thrombi. Because thrombin is the most potent activator of platelets, inducing platelet adherence, aggregation, secretion, and lipid synthesis, interference with its platelet actions could establish a new avenue in cardiovascular therapeutics. Importantly, a thrombin receptor antagonist would block thrombin's action only at the cellular level, not amidst the whole blood coagulation cascade. Thus, it could have an advantage over a direct thrombin inhibitor, which would block all thrombin-mediated processes and offer the risk of untoward bleeding side effects. Indeed, a very attractive feature of a thrombin receptor antagonist, as an antithrombotic drug, is its potential for having just minimal bleeding side effects at therapeutically efficacious dose levels.

Over the past decade, a large body of research on PARs has provided understanding on how thrombin invokes cellular signals and biological events through its action on PARs (Barry et al. 2006; Chackalamannil 2006; Maryanoff 2006). Although thrombin can activate three of the four known PARs, PAR₁, PAR₃, and PAR₄, it is PAR₁ that represents the primary thrombin-responsive receptor in the platelets of humans and primates (Coughlin 2005). Since the discovery of PAR₁ and PAR₄ in human platelets, there has been a keen interest to dissect the specific involvement of each receptor in the process of platelet aggregation. There is a considerable species specificity among the PARs on platelets, which adds to the challenge of developing

a PAR₁ antagonist for use as a therapeutic agent. In contrast to human platelets, which express PAR₁ and PAR₄, murine platelets are sensitive to thrombin via tightly coupled PAR₃ and PAR₄. Transgenic mice that are deficient in these receptors have been used to probe the contributions of different thrombin-sensitive PARs in platelet physiology (Connolly et al. 1996; Darrow et al. 1996; Nakanishi-Matsui et al. 2000; Sambrano et al. 2001). Platelets isolated from PAR₃-deficient mice are less sensitive to thrombin stimulation compared to wild-type platelets, which corresponds to the higher concentrations of thrombin needed to activate PAR₄ in human platelets (Kahn et al. 1998a,b). Apparently, PAR₃ facilitates the cleavage and activation of PAR₄ and is devoid of a signaling sequence within its short intracellular C-terminus. However, PAR₄ deficiency in murine platelets renders these platelets totally unresponsive to thrombin, consistent with PAR₄ being the primary thrombin-sensitive receptor, with PAR₃ serving more as a cofactor (Sambrano et al. 2001). In vivo studies with mice deficient in PAR₄ indicated that disabling this primary thrombin-sensitive PAR provides protection from arterial thrombosis (Sambrano et al. 2001), which is consistent with observations from the blockade of PAR₁ on monkey platelets, where PAR₁ is the primary thrombin-sensitive receptor (as in humans) (Derian et al. 2003b). Guinea pig platelets express three PARs that yield two fully functional systems, PAR₁ and PAR₃/PAR₄ (Cook et al. 1993; Kinlough-Rathbone et al. 1993; Connolly et al. 1994; Derian et al. 1995). Such PAR profiles vary across diverse species (Cook et al. 1993; Kinlough-Rathbone et al. 1993; Connolly et al. 1994; Derian et al. 1995), and thrombin's prothrombotic capacity is dependent on the PAR profile. From this information, it appears that PAR₁ antagonism would furnish sufficient antithrombotic efficacy, with restrained bleeding liability, in humans.

The knowledge platform on thrombin receptors, and on PARs in general, offers a sound basis for the design, discovery, and development of thrombin receptor antagonists for medical applications. However, one crucial issue in this endeavor is the challenge of competing against a native tethered ligand with a small-molecule ligand. In the energetics of receptor affinity, the tethered ligand clearly has a strong entropy advantage because of its *intramolecular* binding. To be an effective therapeutic agent, a thrombin receptor antagonist must possess suitable binding kinetics to abolish the real-world effects of thrombin in vivo. Another issue for the discovery of PAR₁ antagonists as antithrombotic drugs is the problematic preclinical logistics associated with the species variability of PAR₁ in platelets. Since PAR₁ is present in human and monkey platelets, but not in the platelets of mice, rats, or dogs, which are standard animal models for preclinical antithrombotic studies, drug researchers have resorted to using guinea pigs for antithrombotic experiments. However, the presence of both PAR₁ and PAR₃/PAR₄ systems in guinea pig platelets (*vide supra*) means that they will not correspond directly with monkey or human platelets (PAR₁ and PAR₄). In any case, one can also utilize rodents to evaluate a PAR₁ antagonist for nonplatelet pharmacology, such as blockade of the proliferation of smooth muscle cells. Subsequently, guinea pigs and nonhuman primates would be utilized for antithrombotic assessment.

Despite the challenges, PAR₁ has been an exciting drug target in the pharmaceutical industry since the mid-1990s. There has been considerable progress in the discovery

and pharmacological characterization of potent, selective PAR₁ antagonists with both peptidomimetic and nonpeptide structures (Ahn et al. 2003; Derian et al. 2003a,b; Barry et al. 2006; Chackalamannil 2006; Maryanoff 2006; Luo et al. 2007; Meadows and Bhatt 2007; Sokolova and Reiser 2007). Importantly, specific PAR₁ antagonist molecules from Schering-Plough (Sch 530348) and Eisai (E-5555) have advanced into human clinical studies (Anon. 2006, 2007a, b). In this chapter, we will discuss the key medicinal chemistry, pharmacology, and clinical aspects associated with PAR₁ antagonists.

Relative to PAR₄, there has been comparatively much less progress, especially in the development of PAR₄ antagonists. However, lipidated peptide antagonists, known as pepducins, as well as a few other peptide and nonpeptide PAR₄ antagonists, have been identified. This PAR₄ topic will be discussed as well.

12.2 Proteinase-Activated Receptor-1 (PAR₁) Modulators

The initial cloning of PAR₁ in 1991 (Vu et al. 1991a) led to various structural and function studies. PAR₁ is a member of the GPCR superfamily with seven transmembrane (TM) domains, a long extracellular N-terminus (amino acids 1–95), and a standard cytoplasmic C-terminus. Unlike most GPCRs, PAR₁ carries its own ligand within the receptor N-terminus, which is revealed on activation by thrombin. Thrombin cleaves between Arg⁴¹ and Ser⁴² in the amino acid sequence LDPR/SFLLRNPNDKYEPF (Vu et al. 1991a, b; Hung et al. 1992a, b; Coughlin 2000) to unmask a new receptor N-terminus that acts as a “tethered ligand” to activate the receptor. Synthetic peptides with sequences related to the tethered ligand can activate the receptor independently of thrombin. The 14-amino-acid peptide SFLLRNPNDKYEPF-NH₂, which mimics the sequence of the N-terminal portion of the tethered ligand, was first identified as a full agonist for activating both the cloned receptor (expressed in *Xenopus laevis* oocytes) and native receptor on human platelets (Vu et al. 1991a). The maximal response to this agonist peptide in PAR₁ expressing oocytes was comparable to the maximal response to thrombin, as measured by ⁴⁵Ca²⁺ release. The structure–activity relationships (SARs) for thrombin-receptor-activating peptides (TRAPs) have been extensively studied and peptide antagonists have been developed from that information. Subsequently, peptide-mimetic and nonpeptide antagonists were devised (vide infra).

12.2.1 Peptide Agonists and Antagonists

A minimum sequence of five amino acids, as in SFLLR-NH₂ (TRAP-5), is required to have an effective PAR₁ agonist peptide. TRAP-6 (SFLLRN-NH₂) is just slightly more potent than TRAP-5, whereas SFLL-NH₂ has markedly decreased agonist activity (Scarborough et al. 1992). A standard alanine scan of the SFLLR sequence

identified the amino acids that are critical for receptor function (Scarborough et al. 1992; Ceruso et al. 1999). The most crucial residue for agonist activity was found to be the phenylalanine at position 2. Moderately important structural units are a free N-terminal amino group, a basic or aromatic amino acid at position 5, and a bulky aliphatic amino acid at position 4 (Chao et al. 1992; Sabo et al. 1992; Scarborough et al. 1992; Vassallo et al. 1992; Feng et al. 1995; Natarajan et al. 1995). As a correlate, substitution of alanine at position 2 of the agonist peptide domain on PAR₁ selectively abolished receptor activation by thrombin (Scarborough et al. 1992). In a “proline scan” of TRAP-6 or TRAP-5, the amino acids at positions 1 and 3 could be replaced by proline without a major impact on agonist potency (Chao et al. 1992; Sabo et al. 1992; Scarborough et al. 1992; Vassallo et al. 1992; Feng et al. 1995; Natarajan et al. 1995; Seiler et al. 1996; Ceruso et al. 1999). However, simple *N*-methyl substitution at any position of the native pentapeptide was not well tolerated (Feng et al. 1995, Ceruso et al. 1999). Interestingly, one of these derivatives, [*N*-(Me)-S]-FLLR-NH₂, exhibited mixed agonist–antagonist activity, with an EC₅₀ of 16 μM in platelet aggregation, but an IC₅₀ in PAR₁ binding of 0.7 μM (Ceruso et al. 1999). For TRAP-6, the amino acids at position 1, 3, and 6 can be replaced by alanine without significant attenuation of agonist potency, but alanine substitution at positions 2, 4, and 5 are unfavorable. A collection of pentapeptides with agonist data is presented in Table 12.1. Further optimization of TRAP-6 was achieved by substitution of positions 2 and 3 with unnatural amino acid-containing basic side chains. Increased potency occurred when the phenyl ring of the phenylalanine at position 2 had electron-withdrawing aromatic substitution, such as 4-chloro-, 3,4-dichloro-, and 4-fluoro-, with the latter showing the greatest potency enhancement. The opposite effect was observed when the phenyl was substituted with an electron-donating group, such as 2-methoxy or 4-amino

Table 12.1 Agonist peptide analogs of TRAP-5

Peptide	Platelet aggr ^a	Receptor binding ^b
	EC ₅₀ (μM)	IC ₅₀ (μM)
SFLLR-NH ₂	0.49 ± 0.08	1.5 ± 0.5
AFLLR-NH ₂	0.74 ± 0.16	0.66 ± 0.15
SALLR-NH ₂	>50 (10%)	>100
SFALR-NH ₂	1.2 ± 0.3	0.75 ± 0.2
SFLAR-NH ₂	3.4 ± 0.3	1.8 ± 0.8
SFLLA-NH ₂	34 ± 7	1.7 ± 0.7
PFLLR-NH ₂	0.59 ± 0.14	0.52 ± 0.15
SPLLR-NH ₂	>50 (3%)	>100
SFPLR-NH ₂	4.1 ± 0.5	1.7 ± 0.2
SFLPR-NH ₂	>50 (4%)	52 ± 8
SFLLP-NH ₂	>50 (11%)	14 ± 4

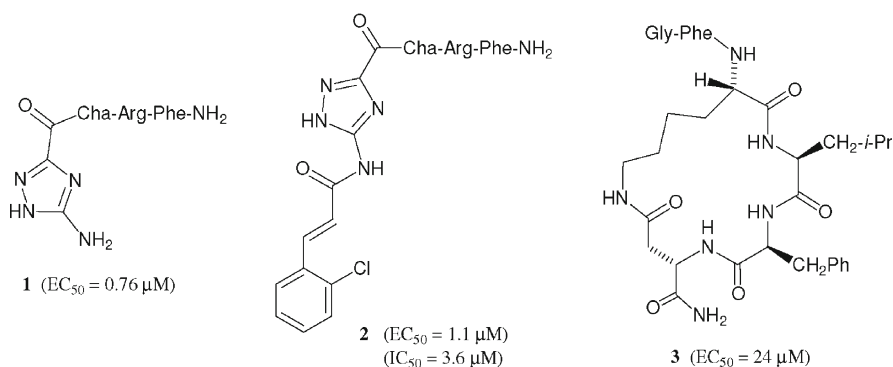
These data are taken from Ceruso et al. (1999).

^aActivation of human platelet aggregation, or percent aggregation induced at 50 μM.

^bInhibition of the binding of radiolabeled agonist peptide [S-Phe(4-F)-hArg-L-hArg-K-(³H-Tyr)-NH₂] to PAR₁ (hArg, homoarginine).

(Nose et al. 1993; Feng et al. 1995; Seiler et al. 1996). Modified hexapeptide A-Phe(4-F)-R-Cha-hArg-Y-NH₂ (Cha, cyclohexylalanine; hArg, homoarginine) was identified as a very potent PAR₁ agonist with an EC₅₀ of 0.01 μM in a platelet-rich plasma (PRP) aggregation assay (Feng et al. 1995). A radioligand for receptor binding studies was obtained by installing ¹²⁵I into A-Phe(4-F)-R-Cha-hArg-Tyr(3-I)-NH₂ (EC₅₀ = 0.03 μM) (Feng et al. 1995). Systematic study of nonproteogenic amino acids at the second and third positions of SFLLR-NH₂ led to potent agonist S-Phe(4-F)-Phe(4-Gn)-LR-NH₂ (Gn, guanidine), with an EC₅₀ of ~0.04 μM for stimulation of human platelet aggregation, which is ~tenfold more potent than the natural pentapeptide (Bernatowicz et al. 1996).

Other interesting structure–activity studies of PAR₁ agonist peptides involved the inclusion of heterocyclic (McComsey et al. 1999a), pseudopeptide (Ceruso et al. 1999), and macrocyclic groups into the peptide motif (Nose et al. 1993; McComsey et al. 1999b). McComsey et al. (1999a) synthesized a series of heterocycle-peptide hybrids with various heterocyclic groups attached to the N-terminus of a relevant tripeptide segment. Several derivatives behaved as full PAR₁ agonists, especially compounds with an aminotriazole group (e.g., **1**) showed similar potency as TRAP-6 in term of stimulation of platelet aggregation (McComsey et al. 1999a). Interestingly, an arylothenoyl unit on the N-terminus afforded analogs with mixed agonist–antagonist activity (e.g., **2**). Earlier studies reported by Bernatowicz et al. (1996) had shown that introduction of a cinnamoyl group at the N-terminus of PAR₁ peptides provided receptor antagonists. The macrocyclic compound cyclo(KFLLR), in which the side-chain amine of lysine is linked to the α-carboxyl of arginine, had comparable activity to SFLLR-NH₂ (Matsoukas et al. 1996). Other macrocyclic derivatives (e.g., **3**, EC₅₀ = 24 μM) were generally much less potent in platelet aggregation compared with SFLLRN-NH₂ (Nose et al. 1993; McComsey et al. 1999b).



The C-terminal sequence of the human adenosine 5'-diphosphate (ADP) receptor P2Y₁, a G_q-coupled GPCR that activates platelets, is also responsible for PAR₁/G-protein binding (Mao et al. 2008). Mao et al. (2008) derived a novel PAR₁ agonist peptide, TFRRLSATR, from this sequence and found that the dose-dependent platelet aggregation induced by it is abolished by the PAR₁ specific antagonist BMS-200261 (vide infra). This TFRRLR-peptide was selective in that its agonist action was not blocked by

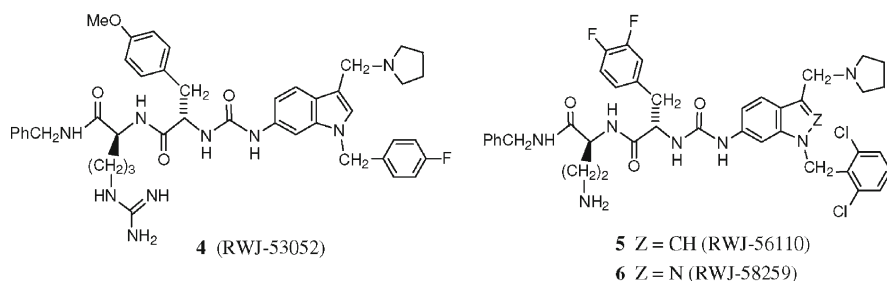
a P2Y₁ antagonist or a thromboxane receptor antagonist. Furthermore, this peptide triggered the G_q-signaling pathway and caused PAR₁ (but not PAR₄) desensitization. These results are not surprising in that the pentapeptide sequence TFRRR is quite analogous to SFLLR. Indeed, consistent with the SAR studies on PAR₁ agonist peptides, the second (Phe) and fifth (Arg) residues of the TFRRR-peptide were the most important.

The development of peptide-based thrombin receptor antagonists was initiated by modifying the PAR₁ tethered-ligand sequence. Scarborough and colleagues disclosed the earliest report on peptide-based PAR₁ antagonists, which capitalized on a 3-mercaptopropionyl (Mpa) group at the N-terminus (Scarborough et al. 1994). Later on, the related peptide Mpa-F-Cha-Cha-RKPNDK-NH₂ was found to block the binding of a radiolabeled agonist peptide to PAR₁ in platelet membranes with a moderate IC₅₀ value of 21 μM (Seiler et al. 1995; Ahn et al. 1997; Kawabata et al. 1999). This compound was capable of preventing both thrombin-induced and SFLLR-induced calcium mobilization and platelet aggregation (Seiler et al. 1995; Ahn et al. 1997; Kawabata et al. 1999). However, Kawabata et al. (1999) determined that this Mpa-peptide is not only a PAR₁ antagonist, but also a PAR₂ partial agonist, by examination of it with HEK293 cells co-expressing PAR₁ and PAR₂. Other peptide antagonists were obtained by modifying the N-terminus of the Mpa-peptide, to achieve similar antagonist potencies (Fujita et al. 1999; Kato et al. 1999). Seiler and coworkers were able to devise more potent peptide-based antagonists (Seiler et al. 1995; Bernatowicz et al. 1996; Seiler and Bernatowicz 2003). Their strategy involved optimization of the agonist activity and then converting the best peptides into antagonists by modifying the N-terminus. Agonists with enhanced potency had nonproteinogenic amino acids substituted for the second and third residues of SFLLR, and antagonists with modest potency had Ser replaced with the Mpa group. Most notably, in exploring a series of *N*-acyl tetrapeptide antagonists, they identified *N*-[(*E*)-cinnamoyl]-Phe(4-F)-Phe(4-Gn)-LR-NH₂ (BMS-197525) as a high-affinity PAR₁ antagonist in a radioligand binding assay (IC₅₀ = 8 nM). BMS-197525 had correspondingly potent inhibition of SFLLRNP-induced platelet aggregation (IC₅₀ = 0.20 μM). Subsequently, BMS-197525 served as a basis for the design of potential photoaffinity tags for PAR₁ (Elliott et al. 1999).

A more potent PAR₁ antagonist emerged by extending the C-terminus of BMS-197525 by one Arg to give *N*-[(*E*)-cinnamoyl]-Phe(4-F)-Phe(4-Gn)-LRR-NH₂ (BMS-200261). This analog had potent IC₅₀ values of 7.5 nM in [³H]SFLRR-NH₂ binding and 21 nM in PAR₁ agonist peptide-induced platelet aggregation. Replacement of the (*E*)-cinnamoyl group of BMS-200261 with a 3-phenyl-2-propynoyl group yielded the tightest binding antagonist, *N*-[PhC≡CC(O)]-Phe(4-F)-Phe(4-Gn)-LRR-NH₂ (BMS-201516), with an IC₅₀ of 4 nM in radioligand binding (albeit 40 nM in agonist-induced platelet aggregation). BMS-200261 was shown to be a selective PAR₁ antagonist relative to PAR₂ (O'Brien et al. 2000) and PAR₄ (Kahn et al. 1999; Quinton et al. 2004). However, this compound had limited effectiveness against thrombin-induced PAR₁ activation (Seiler et al. 1995; Bernatowicz et al. 1996; Seiler and Bernatowicz 2003), which indicates insufficiently robust PAR₁ binding kinetics to compete against the native tethered ligand.

12.2.2 Peptidomimetic Antagonists

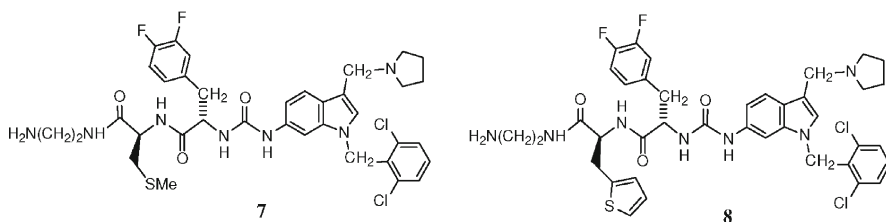
The de novo design of peptidomimetic PAR₁ antagonists originated in our research group as part of collaboration with scientists at COR Therapeutics, Inc. After fruitless screening of a 250K compound library, we turned to a design approach based on pseudopeptides and peptide mimetics related to the SFLLR agonist motif. This effort capitalized on structure–function data for PAR₁ agonist peptides, spatial constraints of key groups in SFLLR, and a heterocyclic template to display desired functional groups. A pharmacophore model was constructed by analyzing SFLLRN α -helical, β/γ -turn, and β -sheet conformations, by applying molecular dynamics searches to energy-minimized structures. Thus, a “three-point model” was obtained to define the range of distances between *amino*, *phenyl*, and *guanidino* groups (Andrade-Gordon et al. 1999; Maryanoff et al. 2003; Maryanoff 2006). A 6-aminoindole was selected as one of the scaffolds (among several) to be used to spatially display the three key functional groups in SFLLR. PAR₁ antagonist lead (RWJ-52021) exhibited an IC₅₀ values of 0.7 μ M in the radioligand binding assay and 0.3/1.3 μ M in blocking platelet aggregation induced by TRAP-6/thrombin (Zhang et al. 2001). Importantly, this compound was effective when thrombin was the platelet agonist, reflecting potential for use of such an agent under native conditions (in vivo or ex vivo). Further optimization of the series led to compounds with improved potency in both TRAP-6-induced and thrombin-induced human platelet aggregation assays. Indole-based analog RWJ-53052 (**4**) was found to inhibit human platelet aggregation induced by both TRAP-6 (IC₅₀ = 0.49 μ M) and thrombin (IC₅₀ = 2.0 μ M); however, it had modest PAR₁ binding affinity (Andrade-Gordon et al. 1999). It was gratifying to have obtained antagonist activity against thrombin, since that reflected favorable binding kinetics relative to the native tethered ligand.



To arrive at more potent antagonists rapidly, we developed robust solid-phase parallel syntheses (Maryanoff et al. 2003; Maryanoff 2006). Consequently, RWJ-56110 (**5**) was identified as potent PAR₁ antagonist: it inhibited platelet aggregation induced by both TRAP-6 and thrombin with IC₅₀ values of 0.34 and 0.16 μ M, respectively, and had reasonable PAR₁ affinity (IC₅₀ = 440 nM) (Andrade-Gordon et al. 1999; Zhang et al. 2001). This compound also inhibited calcium influx in human and rat smooth muscle cells (SMCs) and blocked rat SMC proliferation, and intravenous administration to guinea pigs dose-dependently inhibited

ex vivo platelet aggregation (Andrade-Gordon et al. 1999). However, with respect to in vivo studies, this compound unfortunately had an unexpected hypertensive effect at 7 mg kg⁻¹, i.v., in guinea pigs (Andrade-Gordon et al. 1999). This hurdle was overcome by replacing the indole group with an indazole group to generate bioisostere RWJ-58259 (**6**) (Zhang et al. 2001). This compound had IC₅₀ values of 0.11 and 0.37 μM against TRAP-6-induced and thrombin-induced human platelet aggregation. In an ex vivo guinea pig platelet aggregation model, this compound fully inhibited thrombin-induced (2 U mL⁻¹) platelet aggregation at an intravenous dose of 0.3 mg kg⁻¹. Notably, intravenous administration of RWJ-58259 to anesthetized guinea pigs did not perturb normal hemodynamics (Andrade-Gordon et al. 1999).

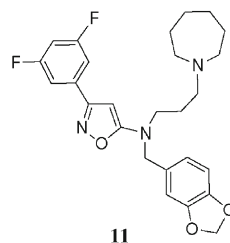
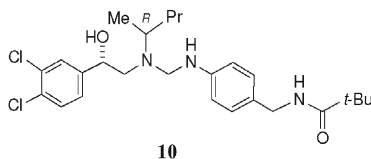
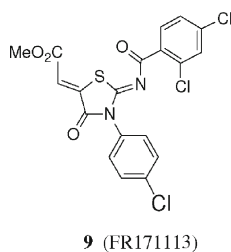
Although RWJ-58259 was not active in two standard guinea pig thrombosis models (arteriovenous shunt and Rose Bengal photoactivation assay), it did block thrombin-induced calcium mobilization and cell proliferation in the rat endothelial cells and SMCs (which contain mainly PAR₁). More importantly, RWJ-58259 showed significant reduction of neointima thickness in a rat restenosis model involving balloon injury after perivascular administration for 14 days, thereby establishing the proof-of-concept that a thrombin receptor antagonist could have therapeutic utility in treating vascular disorders such as restenosis and atherosclerosis (Andrade-Gordon et al. 2001). Given the differential expression of PAR₁ across species (Derian et al. 1995), RWJ-58259 was further evaluated in cynomolgus monkeys in an electrolytic injury thrombosis model. Intravenous infusion of RWJ-58259 prolonged the time to occlusion in an injured carotid artery model. Ex vivo platelet aggregation measurements indicated complete PAR₁ inhibition under these conditions. In the drug-treated group, not only was the thrombus size reduced, but also the composition of the thrombus indicated a switch from platelet-rich to platelet-poor, consistent with the antiplatelet action of a PAR₁ antagonist. This protection against thrombus formation in an arterial injury model was the first robust proof-of-principle study for a PAR₁ antagonist as an antithrombotic agent, and it lent support for potential utility in humans (Derian et al. 2003a).



A next generation of indole-based peptide mimetics, bearing a basic amine at the C-terminus, was developed later (Zhang et al. 2003). Several compound showed very high affinity for the thrombin receptor. For example, compounds **7** and **8** had IC₅₀ values in the PAR₁ radioligand binding assay of 25 and 35 nM, respectively, and potent inhibition of TRAP-6-induced platelet aggregation, with IC₅₀ values of 7–80 nM (Zhang et al. 2003). However, their activity against thrombin-induced platelet aggregation did not reflect a meaningful boost in potency (IC₅₀ = ca. 250 nM).

12.2.3 Nonpeptide Antagonists

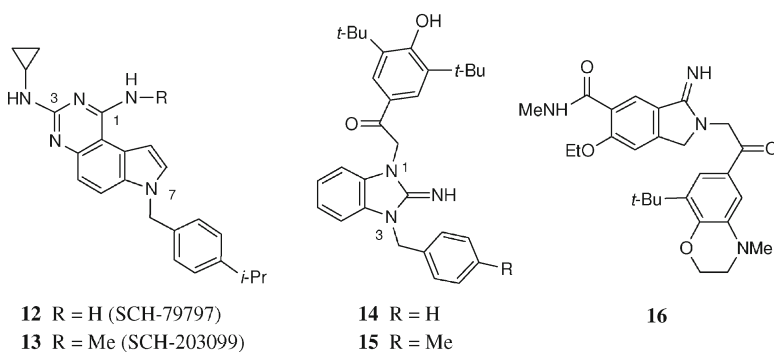
FR171113 (**9**) was the first nonpeptide PAR₁ antagonist to be reported in the scientific literature (Kato et al. 1999). This compound showed potent inhibitory activity in TRAP-6-induced human platelet aggregation ($IC_{50} = 0.15 \mu M$), and no inhibitory effect on ADP-induced and collagen-induced platelet aggregation in human PRP up to 100 μM . FR171113 appeared to be a specific inhibitor to PAR₁ and had no inhibitory effect on PAR₂ and PAR₄ in calcium-influx assays and PAR₄ peptide-induced platelet aggregation (Kato et al. 2003). It also appeared to act directly on PAR₁, rather than as a direct thrombin inhibitor (Kato et al. 1999). FR171113 inhibited TRAP-6-induced and thrombin-induced aggregation of guinea pig platelets in a dose-dependent manner in vitro with IC_{50} values of 1.5 and 0.35 μM , respectively. Ex vivo, subcutaneous administration of FR171113 (0.1–3.2 mg kg⁻¹) inhibited PAR₁ agonist peptide-induced platelet aggregation with an ED_{50} of 0.49 mg kg⁻¹, while there was no significant inhibition when ADP and collagen were used as agonists. The compound significantly inhibited FeCl₃-induced thrombus formation in guinea pig carotid artery. The time of thrombotic occlusion for doses of 0.32, 1.0, and 3.2 mg kg⁻¹ (s.c.) was 31, 45 ($p < 0.05$), and 93 ($p < 0.01$) min, respectively, whereas the time to occlusion in the vehicle-control group was 5–18 min. At a tenfold higher dose of 32 mg kg⁻¹, s.c., there was no prolongation of bleeding time (Kato et al. 1999).



Barrow et al. reported urea-based and phenylisoxazole-based small-molecule series with PAR₁ antagonist activity (Barrow et al. 2001; Nantermet et al. 2002), several of which exhibited submicromolar IC_{50} values in an assay involving TRAP-peptide-induced secretion of [³H]5-hydroxytryptamine (5-HT) from washed human platelets. Starting from a sizable tris-urea compound with an IC_{50} of 0.6 μM from the screening of combinatorial libraries, they arrived at mono-urea derivatives, such as **10**, which had an IC_{50} of 0.23 μM in the [³H]5-HT secretion assay, as well as an IC_{50} of 0.12 μM in a PAR₁ radioligand binding assay. In the isoxazole series, a structurally novel lead of moderate potency ($IC_{50} = 9 \mu M$ in 5-HT secretion) led to some relatively potent compounds, such as **11**, with IC_{50} values of 0.09 μM in 5-HT secretion and 0.15 μM in PAR₁ radioligand binding (Nantermet et al. 2002). Although various analogs had limited potency in blocking thrombin-induced [³H]5-HT release ($IC_{50} > 1.0 \mu M$), **11** had an IC_{50} of 0.51 μM , without affecting thrombin enzymatic activity or other agonist-induced platelet activation (collagen

or ADP). At 4 μM , **11** completely blocked platelet aggregation induced by 1 nM thrombin for 10 min.

The pyrroloquinazoline SCH-79797 (**12**), and its *N*-methyl analog (SCH-203099, **13**), are noteworthy nonpeptide PAR_1 antagonists (Ahn et al. 1999). The parent compound for this series came from high-throughput screening (HTS) of compound libraries. The substituted pyrroloquinazolines showed a very specific SAR with the following preferences for good affinity: 4-isopropylbenzyl group at N7, a free amino group at C1, and a substituted amino group at C3. A cyclopropylamino group at C3 was optimal, as seen in **12** and **13**, which inhibited the binding of the high-affinity TRAP, A-Phe(4-F)-R-Cha-hArg- $[\text{^3H}]$ Tyr-NH $_2$ ($[\text{^3H}]$ ha-TRAP), to PAR_1 in human platelet membranes (Ahn et al. 1997) with IC_{50} values of 70 and 45 nM, respectively (Ahn et al. 2000). Analysis of saturation binding of $[\text{^3H}]$ ha-TRAP in the presence and absence of SCH-79797 indicated that competitive inhibition of PAR_1 . SCH-79797 and SCH-203099 blocked platelet aggregation induced by ha-TRAP in a concentration-dependent fashion, with IC_{50} values of 300 nM and 150 nM, and inhibited aggregation induced by α -thrombin with IC_{50} values of 3 μM and 700 nM, respectively. These compounds did not block platelet aggregation induced by PAR_4 agonist peptides, γ -thrombin, ADP, or collagen. Binding of these compounds to platelets was reversible, but full reversal of inhibition required washing for 20 min. These two compounds had no agonist activity at concentrations as high as 3 μM , nor did they inhibit the catalytic activity of thrombin. SCH-79797 inhibited calcium transients induced by thrombin (3 nM) and the peptide agonist TFLLRNPNDK-NH $_2$ with K_i values of 82 and 55 nM, respectively, but it had no effect on PAR_2 -induced calcium influx in human coronary artery SMCs. SCH-79797 inhibited the proliferation of human coronary artery SMCs induced by PAR_1 agonist peptide and thrombin, as measured by $[\text{^3H}]$ thymidine incorporation. While the collections of data indicate that SCH-79797 and SCH-203099 are potent, selective, PAR_1 antagonists, they were relatively weak in inhibiting thrombin-induced human platelet aggregation.



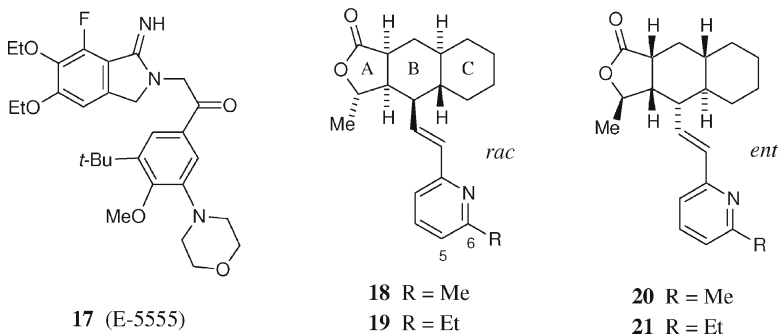
SCH-79797, which is commercially available as a tool compound, was found to exhibit antiproliferative and antiangiogenic effects, block cell migration, and reduce myocardial ischemia/reperfusion injury in different laboratories (Ma et al. 2005; Zania et al. 2006; Kaufmann et al. 2007; Strande et al. 2007). Recently, Di

Serio et al. reported that SCH-79797 inhibits serum-dependent cell growth in vitro in three different tumor cell lines (NIH3T3, HEK293, and A375). However, this antiproliferative effect may not be mediated by PAR₁ antagonism, since the same effect was observed in embryonic fibroblasts derived from PAR₁ null mice (Di Serio et al. 2007). These results may raise a concern about off-target effects from this and other PAR₁ antagonists when being applied to the elucidation of PAR₁-mediated cellular function.

A series of 2-aminobenzimidazole derivatives was identified as PAR₁ antagonists from a different HTS hit (Chackalamannil et al. 2001). Systematic variation of the N3 substituent revealed that a lower alkyl or benzyl yields optimal potency. Compounds **14** and **15**, bearing N3 benzyl groups, gave IC₅₀ values of 65 and 33 nM, respectively, against PAR₁ agonist peptide binding. These compounds had moderate potency vs. ha-TRAP-induced (e.g., **14**: IC₅₀ = 265 nM) and thrombin-induced (e.g., **14**: IC₅₀ = 600 nM) human platelet aggregation (Chackalamannil et al. 2001).

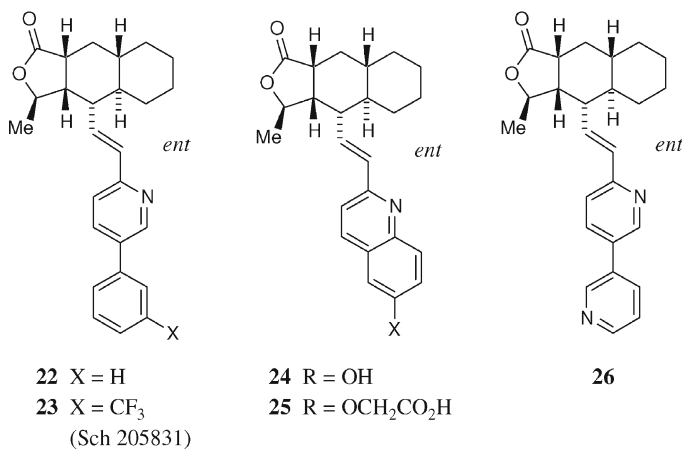
Kawahara et al. (2004) reported on an orally active, nonpeptide PAR₁ antagonist at a scientific meeting in 2004. An interesting small-molecule PAR₁ antagonist obtained from HTS with a PAR₁ binding assay was ER-97719-15, which is actually compound **14**. Optimization around this structure afforded three interesting compound series. One series encompassed derivative ER-129614-06 (**16**), which had IC₅₀ values of 14 and 28 nM against thrombin-induced and TRAP-induced platelet aggregation, respectively. Oral administration of ER-129614-06 at 100 mg kg⁻¹ in a guinea pig arterial thrombosis model resulted in a significant anti-thrombotic effect.

Diverse PAR₁ antagonists based on various cyclic guanidines and amidines, including ER-129614-06, were disclosed by researchers at Eisai Co. in massive patent applications (Suzuki et al. 2002a–c). Although limited data are available publicly in print on clinical candidate E-5555 (**17**), preclinical data were presented at a key scientific meeting in November 2003 (Kogushi et al. 2003). In a radioligand binding assay, E-5555 had an IC₅₀ value of 19 nM. Its IC₅₀ values in TRAP-induced and thrombin-induced platelet aggregation were 31 and 64 nM, respectively. E-5555 selectively inhibited TRAP- and thrombin-induced guinea pig PRP aggregation with IC₅₀ values of 97 and 130 nM, respectively. In a guinea pig photochemically induced thrombosis model, E-5555 inhibited thrombus formation dose-dependently, without prolonging bleeding time at a dose level 30-fold higher than the minimum effective dose of 30 mg kg⁻¹ (Kogushi et al. 2003). More recently, it was reported that E-5555 inhibits the release of sCD40L induced by thrombin and TRAP (IC₅₀ = 47 and 38 nM, respectively), without affecting ADP-induced sCD40L release. E-5555 also inhibited the thrombin-stimulated release of IL-6 from human coronary artery SMCs (IC₅₀ = 0.19 nM) and the P-selectin expression on human coronary artery endothelial cells (IC₅₀ = 16 nM) (Kogushi et al. 2007). A randomized, double-blind, placebo-controlled study of E-5555 in humans is in progress, with an expected enrollment of 600 subjects, to evaluate the efficacy and safety of E-5555 in patients with coronary artery disease (Anon 2006).



An exciting chemical series of PAR₁ antagonists is based on the structure of the natural product himbacine, a piperidine alkaloid isolated from the bark of Australian magnolias (Chackalamannil 2006). Interest in himbacine was originally connected with its antimuscarinic properties as a potential treatment for Alzheimer's disease. In a total synthesis project on himbacine, several analogs were synthesized (Chackalamannil et al. 1996, 1999; Doller et al. 1999), and one of these, **18** (as a racemate), turned out later to be a PAR₁ antagonist lead in an HTS campaign (IC₅₀ of 300 nM in PAR₁ radioligand binding) (Chackalamannil et al. 2005). By replacing the piperidine unit in himbacine with a less basic pyridine unit, activity against muscarinic receptors was eliminated, such that the PAR₁ antagonism was comparatively selective. Initial SAR studies involved optimizing the substitution pattern on the pyridine ring and alkyl substitution at C6 (R) became preferred over alkyl groups at other positions. Ethyl at C6 (**19**) gave the best PAR₁ affinity, with an IC₅₀ value of 85 nM. The *ent*-himbacine stereoisomers of **18** and **19** (viz. **20** and **21**) were much more potent (IC₅₀ = 150 and 20 nM) than the himbacine stereoisomers (IC₅₀ = 1,500 and 400 nM). Studies with additional analogs established that the *ent*-himbacine absolute chirality is crucial for potent PAR₁ antagonism. Compound **21** inhibited human platelet aggregation induced by haTRAP with an IC₅₀ of 70 nM. In a cynomolgus monkey ex vivo platelet aggregation model, administration of **21** by intravenous infusion (10 mg kg⁻¹, 30 min) gave nearly complete inhibition of platelet aggregation induced by haTRAP in the PRP drawn from the drug-treated group. However, this compound had very low oral bioavailability in rats (*F* < 3%) with a fairly short half-life (*t*_{1/2} < 1 h) (Chackalamannil et al. 2005). Structure modification was carried out to improve the oral bioavailability. Although C5 alkyl substitution and C6 aryl substitution led to diminish PAR₁ antagonist potency, C5 aryl substitution showed promise. The C5 phenyl derivative, **22**, had a binding IC₅₀ of 27 nM and a better rat PK profile. This finding led to C5 phenyl derivatives bearing CF₃ and halogen substituents at the *meta* and *ortho* positions with excellent PAR₁ affinity and good oral bioavailability in rats. A benchmark compound was Sch 205831 (**23**), which had an IC₅₀ of 11 nM (*K*_i = 2.7 nM) in PAR₁ binding and inhibited haTRAP-induced and thrombin-induced aggregation of human platelets with IC₅₀s of 24 and 44 nM, respectively (Chackalamannil et al. 2005). Sch 205831 was potent in α -thrombin-mediated calcium transient experiment (*K*_d = 2.6 nM) and

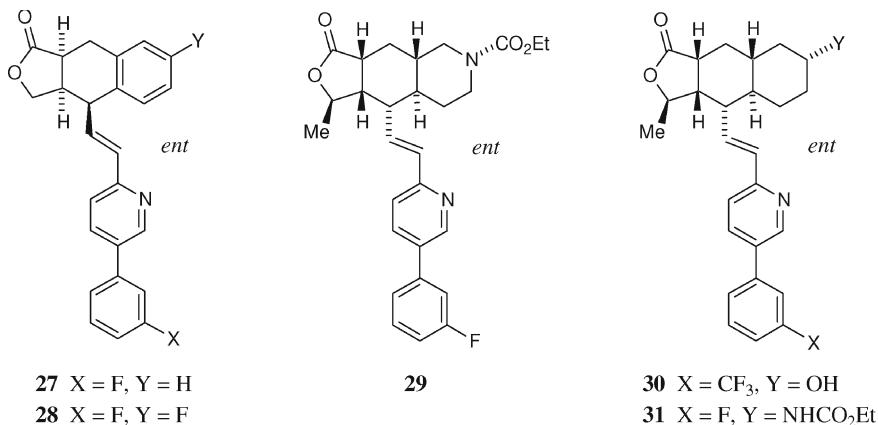
in a proliferation assay ($K_i = 13$ nM) with human coronary artery SMCs. This compound showed 30% oral bioavailability in rats and 50% in monkeys. In the ex vivo platelet aggregation assay in cynomolgus monkey, Sch 205831 showed complete and sustained inhibition of platelet aggregation at 3 mg kg⁻¹ after oral administration. It also showed potent dose-dependent inhibition of platelet deposition on thrombogenic surfaces in an arteriovenous shunt model in baboons after oral administration. Sch 205831 was a selective PAR₁ antagonist. Unfortunately, this compound was found to induce rat liver cytochrome P450 enzymes at high doses (Xia et al. 2007; Clasby et al. 2007a). Sch 205831 is highly hydrophobic (clog $P = 6$) and had slow metabolite clearance in vivo; also, one of its major metabolite had an undesirable off-target activity at the cannabinoid CB2 receptor ($K_i = 38$ nM), which can raise immunological concerns (Pertwee 2000; Klein et al. 2003). Therefore, this compound was not pursued further as a development candidate.



A series of himbacine-related quinoline derivatives was also reported, and several compounds exhibited potent PAR₁ affinity. For example, **24** had an IC₅₀ of 6.3 nM in PAR₁ binding assay. Whereas **24** was not active in the cynomolgus monkey model of ex vivo platelet aggregation on i.v. dosing, congener **25** (binding IC₅₀ = 29 nM) was; indeed, **25** completely inhibited ex vivo platelet aggregation at 2 h after i.v. dosing. Studies were also conducted with various analogs having a modified 2-vinylpyridine segment (Xia et al. 2007). 3-Pyridyl derivative **26** had good PAR₁ affinity (IC₅₀ = 22 nM), good PK properties in rats, and weak affinity for CB2 receptors. Furthermore, it completely inhibited ex vivo platelet aggregation in cynomolgus monkeys 4 h after an oral dose of 3 mg kg⁻¹. Analogs with alterations to the lactone A-ring were also explored, but many of them showed a sharp decrease in PAR₁ binding affinity (Xia et al. 2006).

Additional variations on the himbacine structural motif have been reported (Chackalamannil 2006; Chelliah et al. 2007; Clasby et al. 2007b). One type of structure possessed an aromatic C-ring and lacked a methyl substituent on the A-ring, with the 5-phenylpyridine-substituted analogs giving the greatest PAR₁

antagonist activity (Clasby et al. 2007b). For example, the 5-phenylpyridine derivative with a *meta*-fluoro group (**27**) had an IC_{50} value of 7.5 nM in receptor binding. However, some of the key potent compounds had low in vivo activity due to poor oral bioavailability. In the SAR for substitution of the C-ring, alkyl, alkoxy, carboxyl, and hydroxyl gave reduced potency ($IC_{50} > 100$ nM), whereas halogen and CF_3 substituents gave potent compounds. Potent difluoro analog **28** ($IC_{50} = 7.6$ nM) exhibited robust activity for 24 h in the cynomolgus monkey ex vivo study at an oral dose of 2 mg kg^{-1} , and had excellent blood levels in rats and cynomolgus monkeys. Notably, **28** had a slow rate of dissociation from the receptor ($t_{1/2} = 139$ min), which would possibly afford a better ability to compete with the PAR_1 intramolecular activation mechanism.



Another compound series incorporated heteroatoms into the C-ring of the tricyclic scaffold, resulting in several analogs with excellent PAR_1 affinity, and some compounds also demonstrated good inhibition of platelet aggregation on oral administration in the ex vivo cynomolgus monkey model (Chelliah et al. 2007). A benchmark compound was **29** (PAR_1 binding IC_{50} of 10 nM), which inhibited monkey ex vivo platelet aggregation to the extent of 70% at 24 h after a single oral dose of 3 mg kg^{-1} (Chelliah et al. 2007). The oral bioavailability (F) of **29** in cynomolgous monkeys was 62%, and its i.v. half-life was 6.2 h. Importantly, there were minimal amounts of oxidative metabolites with (M+16) by mass spectrometry, unlike congener **23**, and no undesirable effect on P450 enzyme induction in mice.

Oxidative metabolism occurred on the C-ring of **23** to furnish three major hydroxylated (M+16) derivatives. One of these, compound **30**, turned out to be a potent PAR_1 antagonist with excellent oral bioavailability and a favorable rat liver enzyme-induction profile (Clasby et al. 2007a). It bound to PAR_1 reversibly, but with a very slow dissociation rate. Substitution of the C-ring with other functionalities, such as carboxyl or amine groups, led to carbamate derivative Sch 530348 (**31**) as a key lead compound (Greenlee 2005; Chackalamannil et al. 2008). This compound demonstrated 100% thrombin inhibition in a 24-h period

from an oral dose of 1 mg kg⁻¹, was efficacious in a primate model of arterial thrombosis, and has excellent oral bioavailability ($F = 86\%$) (Greenlee 2005; Chackalamannil et al. 2008).

Phase 1 clinical data on Sch 530348 were reported at a scientific meeting in 2005 (Kosoglou et al. 2005). A randomized, evaluator-blind, placebo-controlled, ascending-dose, parallel-group study was conducted in 50 healthy men receiving oral Sch 530348 (0.25–40 mg) or placebo. The drug caused significant dose-related inhibition of TRAP-induced platelet aggregation (ex vivo) with greater than 90% inhibition at 1 h at the 20- and 40-mg doses. At doses of greater than or equal to 5 mg, such inhibition was maintained for at least 72 h. All doses were well tolerated and adverse events were generally mild and nonspecific. Phase 2 data on Sch 530348 were reported at a scientific meeting in 2007 (Moliterno et al. 2007). A 1,030-patient, multinational, randomized, double-blind, placebo-controlled, dose-ranging TRA-PCI trial assessed 10, 20, and 40 mg initial oral doses, followed by 0.5, 1.0, or 2.5 mg maintenance doses for 60 days. Sch 530348 did not increase bleeding when added to standard antiplatelet therapy for patients undergoing percutaneous coronary intervention; however, this trial was not powered to show efficacy (Anon. 2007a; Moliterno et al. 2007). Sch 530348 is currently undergoing Phase 3 clinical development (Anon. 2007a, b).

12.2.4 Cell-Penetrating Pepducins

The action of “pepducins” on PAR₁ contrasts with the aforementioned small-molecule peptides or nonpeptides (Covic et al. 2002a). Whereas the small molecules modulate PAR₁ by binding to the extracellular domain of the GPCR, the pepducins interfere with PAR₁/G-protein binding on the inside surface of the receptor (Covic et al. 2002a,b). The cell-penetrating pepducins are lipidated peptides based on intracellular loop sequences that target the receptor-G protein interface and have been used to elucidate function roles of PAR₁ and PAR₄ (Covic et al. 2002a, b, 2006; Stampfuss et al. 2003; Hollenberg et al. 2004; Majumdar et al. 2004; Boire et al. 2005; Houle et al. 2005; Kaneider et al. 2005, 2007; Keuren et al. 2005; Leger et al. 2006). The initial design of such peptides emerged from mutagenesis studies, which demonstrated that the third intracellular loop (i3) of a GPCR mediates a large part of the coupling between the receptor and its G-protein (Cotecchia et al. 1992; Kjelsberg et al. 1992; Kostenis et al. 1997). The i3 peptide P1-i3-40, which contains the adjacent transmembrane α -helical amino acids from TM5 of PAR₁ caused a surprisingly rapid intracellular calcium influx instead of inhibiting platelet activation (Covic et al. 2002a,b). A series of progressively truncated forms of P1-i3-40 were prepared to determine whether the N-terminal hydrophobic region was required for PAR₁ agonist activity. The P1-i3-19 peptide, which lacks a hydrophobic N-terminus, did not stimulate calcium influx, whereas the P1-i3-33 peptide retained potency. Lipidation of the

N-terminal of the truncated peptides enhanced activity (Kuliopulos and Covic 2003). For example, the palmitoylated inactive peptide P1-i3-19, namely P1pal-19 (palmitoyl-NH-RCLSSSAVANRSKKSRA₂L₂F-NH₂), was a full agonist of platelet signaling and aggregation. P1pal-19 was also a full agonist of highly homologous PAR₂, and a partial agonist of the cholecystokinin B (CKB) receptor; however, this peptide did not activate PAR₄. Several other derivatives of the PAR₁ i3-loop were screened, and a shorter C-terminal PAR₁ pepducin, P1pal-13, was found to be less active than P1pal-19 as a PAR₁ agonist, but highly selective for PAR₁ (did not cross-activate PAR₂ or CKB expressed in fibroblasts) (Covic et al. 2002a). In contrast, the P1pal-12 pepducin, comprising the N-terminal portion of the i3 loop, lacked agonist activity. However, this peptide was a full antagonist of PAR₁-dependent inositol phosphate production and calcium signaling in platelets and Rat1 fibroblasts expressing PAR₁. Further, it blocked thrombin-induced calcium response and inhibited SFLLRN-induced platelet aggregation (Covic et al. 2002a, b; Stampfuss et al. 2003; Kaneider et al. 2005; Keuren et al. 2005). The peptide had no activity against other platelet surface receptors, including the GPCRs PAR₄, thromboxane, P2Y₁, P2Y₁₂, and the non-GPCRs collagen and von Willebrand factor (Covic et al. 2002b). Kubo et al. (2006) showed that P1pal-19 produced endothelial NO-dependent relaxation in isolated rat aorta and epithelial prostanoid-dependent relaxation in mouse bronchus while P1pal-12 partially inhibited the vasorelaxation induced by both P1pal-19 and TFLLR peptides. P1pal-12 and another full antagonist pepducin, P1pal-7, significantly blocked tumor growth and angiogenesis of breast cancer xenografts in nude mice (Boire et al. 2005).

12.3 Proteinase-Activated Receptor-4 (PAR₄) Antagonists

PAR₄, which was initially identified by Xu et al. (1998), is homologous to PAR₁, as a member of the GPCR superfamily with a long extracellular N-terminus (amino acids 1–95), and a standard cytoplasmic C-terminus. Like PAR₁, PAR₄ has a cryptic ligand within its N-terminus, which is revealed on activation by thrombin. Thrombin cleaves the peptide bond in human PAR₄ between Arg-47 and Gly-48 in the sequence xxxR/GYPGVxxxx to release the “tethered ligand” GYPGQV and activate the receptor (Kahn et al. 1998a,b; 1999 Xu et al. 1998). The synthetic peptide GYPGQV-NH₂ does function as an agonist for PAR₄, but a high concentration of 300–500 μ M is required for activating human platelets; also an EC₅₀ of 30 μ M is required for activating PAR₄ expressed in *Xenopus* oocytes (Kahn et al. 1998b, 1999; Xu et al. 1998; Faruqi et al. 2000). GYPGKF-NH₂, which represents the first six amino acids of the new amino terminus that is unmasked by thrombin cleavage of mouse PAR₄ (Kahn et al. 1998b), is more potent than the cognate human sequence on mouse and human receptors (Kahn et al. 1999b; Covic et al. 2000).

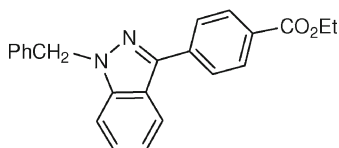
12.3.1 Peptide Agonists

Structure–activity analysis was carried out to define the amino acids that are important for the potency and specificity of PAR₄ agonist activity by using GYPGKF as a starting sequence (Faruqi et al. 2000). While GYPGKF-NH₂ had only 55% of the maximal response triggered by thrombin in PAR₄ expressing cells measured by inositol phosphate release at 500 μM of concentration, alanine or serine substitution at position one of GYPGKF-NH₂ yielded a gain of function for PAR₄ activation. The activity of AYPGKF-NH₂ or SYPGKF-NH₂ increased to 92% of the maximal thrombin response in this assay with an EC₅₀ of 20 or 50 μM, respectively. Threonine substitution at position 1 caused a loss of function. These data point to small aliphatic side chains as being preferred at position 1, in analogy with PAR₁ agonist peptide SAR. Substitution of alanine for the tyrosine at position 2 in GYPGKF-NH₂ resulted in a loss of agonist activity. The replacement of tyrosine with phenylalanine or 4-fluorophenylalanine slightly decreased PAR₄ agonist activity; however, this change greatly increased activity toward PAR₁. This result suggests that the presence of a tyrosine at position two is an important determinant of the activity of GYPGKF-NH₂ and its specificity for PAR₄ over PAR₁. The third and fourth positions at GYPGKF-NH₂ are also important since substitutions at either of these position resulted in substantial loss of PAR₄ agonist activity. The activity of the most potent peptide, AYPGKF-NH₂, was further confirmed in peptide-induced cytoplasmic calcium influx. AYPGKF-NH₂ stimulated calcium mobilization in PAR₁ null fibroblasts transfected with PAR₄ with an EC₅₀ value of 25 μM, whereas more than 200 μM GYPGKF-NH₂ was required to elicit a similar response. At 100 μM, AYPGKF-NH₂ induced platelet ATP release and aggregation, while GYPGKF-NH₂ caused only shape change at 500 μM. AYPGKF-NH₂ was selective for PAR₄ over PAR₁, PAR₂, and PAR₃. Recently, a number of PAR₄ agonist peptides have been evaluated, with AYPGKF-NH₂ still being the most potent and selective one and YAPGKF-NH₂ as a PAR₄ inactive peptide (Hollenberg et al. 2004).

12.3.2 Peptide and Nonpeptide Antagonists

To date, there are only three small molecules reported as PAR₄ antagonists. Two are the peptide-derived compounds (*E*)-cinnamoyl-YPGKF-NH₂ and (*E*)-cinnamoyl-APGKF-NH₂ (Hollenberg and Saifeddine 2001; Ma et al. 2001; Hollenberg et al. 2004). Both *N*-acylated peptide derivatives of the murine PAR₄ tethered ligand were antagonists of both thrombin and the PAR₄ agonist peptides GYPGKF-NH₂ and AYPGKF-NH₂ in a PAR₄-dependent rat platelet aggregation assay without affecting ADP-mediated aggregation. Another one is YD-3 (Wu et al. 2000, 2002, 2003; Peng et al. 2004; Wu and Teng 2006), indazole derivative (**32**), which resulted from antiplatelet screening of the various chemical compounds. In washed rabbit platelets, where there is no PAR₁ expression, YD-3 inhibited platelet aggregation induced by thrombin (0.1 U mL⁻¹) with an IC₅₀ of 28 μM and had a dose-dependent

inhibition of thrombin-mediated inositol phosphate formation. However, in human platelets, where PAR₁ is a major functional thrombin receptor, YD-3 only partially inhibited platelet aggregation by the lower concentration of thrombin (0.05 U mL⁻¹) and had no inhibitory effect on SFLLRN-induced platelet aggregation. In contrast, YD-3 markedly blocked platelet aggregation induced by PAR₄ agonist peptides GYPGKF-NH₂ and AYPGKF-NH₂ with an IC₅₀ of 0.13 μM (Wu et al. 2000; Wu and Teng 2006), and showed a synergistic effect when it was combined with a PAR₁ antagonist in inhibiting platelet activation (Wu and Teng 2006). This compound did not inhibit other platelet agonist-induced aggregation in both rabbit and human platelets (Wu et al. 2000, 2002). Interestingly, the same authors showed that YD-3 significantly inhibited vascular SMC proliferation stimulated by thrombin in vitro and oral administration of YD-3 exhibited a marked reduction in neointimal thickness in balloon injured rat model (Peng et al. 2004). This inhibition of neointimal formation seems the result of inhibiting PAR₁ activity in the vessel (Peng et al. 2004). Several new analogs of YD-3 were reported recently (Chen et al. 2008).



32 (YD-3)

12.3.3 Cell-Penetrating Pepducin

PAR₄-based pepducins have been developed (Covic et al. 2002a), as had been done for PAR₁. P4pal-10 was designed based on the human PAR₄ i3 loop. This peptide had no agonist activity as measured by platelet aggregation, intracellular calcium release in human platelets, or inositol phosphate production in PAR₄ expressing cells. In contrast, P4pal-10 completely blocked PAR₄ peptide AYPGKF-NH₂ and markedly inhibited thrombin-induced aggregation in both human and mouse platelets. Notably, the peptide could also partially block activation of PAR₁ by SFLLRN-NH₂, but did not appreciably inhibit aggregation induced by other platelet agonist thromboxane analog U46619, ADP, collagen, and ristocetin (an antibiotic that stimulates platelet aggregation) (Covic et al. 2002a). P4pal-10 pepducin inhibited platelet aggregation and acted as a PAR₄ antagonist in vivo. The effect of P4pal-10 as a protective agent against systemic platelet activation was evaluated in mice. Intravascular infusion of the platelet agonist mixture resulted in systemic thrombus formation measured by a significant drop of the platelet counts. Pre-infusion of P4pal-10 provided dose-dependent protection of thrombus formation, consistent with the phenotype observed with mice deficient in PAR₄ (Sambrano et al. 2001). Infusion of P4pal-10 also caused unstable homeostasis in mice, as demonstrated by a rebreeding phenotype (Covic et al. 2002a), and prolonged the occlusion time of carotid arteries

(Covic et al. 2006). PAR₄ peptidic antagonists have shown efficacy in other disease models including inflammation (Hollenberg et al. 2004; Boire et al. 2005; Houle et al. 2005; Kaneider et al. 2005).

12.4 Potential Therapeutic Applications

12.4.1 Antithrombotic Agents

It is clear from the diverse array of cells that express thrombin receptors that several potential areas exist where a thrombin receptor antagonist could prove to have therapeutic benefits. However, thrombosis and atherosclerosis/restenosis have continued to occupy center stage with respect to the evaluation of compounds and the development of new drugs.

Thrombosis entails clot formation in the blood vessels, which is primarily caused by fibrin formation and platelet activation. Although, platelet activation and subsequent platelet aggregation play a central role in maintaining normal haemostasis, excessive platelet-rich thrombus formation, especially in high-shear arterial environments, is the major underlying pathology of chronic cardiovascular diseases, such as unstable angina, acute myocardial infarction, and stroke. Platelets are activated by a variety of endogenous agonists, but thrombin is the most potent stimulus known. Work in different research groups has led to the discovery of several small-molecule PAR₁ antagonists with activity in thrombosis models in nonhuman primates. These observations afforded positive proof of concept for PAR₁ antagonists as potential antithrombotic drugs. Today, there are two orally bioavailable PAR₁ antagonists undergoing human clinical evaluation, with E-5555 in Phase 2 and Sch 530348 in Phase 3. Results from these studies will eventually shed light on the benefits of a PAR₁ antagonist in cardiovascular diseases. While a PAR₁ antagonist alone is proven to be effective against thrombosis disorders in nonhuman primates, there is also interest in developing both PAR₁ and PAR₄ antagonists as novel antiplatelet drugs. Although antibodies to the thrombin cleavage site in PAR₄ had no effect on the activation of human platelets by thrombin, platelet activation was markedly inhibited, even with high concentrations of thrombin, when PAR₄ antibodies were combined with a PAR₁ antagonist (Kahn et al. 1999). Treatment with a small-molecule PAR₁ antagonist, or PAR₁ peptidic combined with either a PAR₄ peptidic or a small molecule inhibitor, showed increased efficacy in inhibiting platelet aggregation (Leger et al. 2006; Wu and Teng 2006).

12.4.2 Treatment of Atherosclerosis and Restenosis

PAR₁, the predominant receptor of thrombin in vascular cells, elicits a variety of responses including regulation of vascular permeability, cell migration and proliferation, and angiogenesis (Derian et al. 2003; Coughlin 2005; Tsopanoglou and

Maragoudakis 2007). PAR₁ is upregulated in human atherosclerotic lesions, both in vascular SMCs and inflammatory macrophages (Nelken et al. 1992; McNamara et al. 1993; Wilcox 1994; Wilcox et al. 1994; Harker et al. 1995; Patterson et al. 2001; Coughlin 2005). Elevated levels of thrombin were detected in arterial injury and in neointima of human atherosclerotic lesions (Cheung et al. 1999). Activation of PAR₁ triggers mitogenic responses in SMCs and fibroblasts (McNamara et al. 1993). In addition, thrombin-mediated endothelial and SMC activation results in the secretion of various inflammatory mediators, as well as increased vascular permeability to plasma proteins. Thrombin stimulates adhesion of neutrophils and monocytes to vascular endothelium (McNamara et al. 1993; Herbert et al. 1997). Yet, there is no direct evidence to show that a PAR₁ antagonist is efficacious in atherosclerotic animals or patients. E-5555 has caused a decrease in the release of the inflammatory markers sCD40 and IL-6, which indicates PAR₁ involvement in the inflammatory process that is characteristic of atherosclerotic plaque formation (Kogushi et al. 2007). Evaluation of the safety and efficacy of Sch 530348 vs. standard of care in subjects with a history of atherosclerotic disease is ongoing (Anon 2007b).

One of the complications associated with clinical procedures to reopen vessels is a rebound proliferative response to tissue injury and vessel distension. This restenosis is primarily vascular smooth muscle-dependent in the intimal of the vascular wall (McNamara et al. 1993; Herbert et al. 1997; Coughlin 2001, 2005). Increased expression of PAR₁ during neointimal formation after vascular injury suggests that a PAR₁ antagonist might be beneficial for the prevention of restenosis. An antisense approach was first taken to assess the role of PAR₁ in a rabbit vascular injury model (Herbert et al. 1997). While this treatment led to decreased expression of PAR₁ in the vascular wall, intimal thickening was not affected after injury, suggesting that PAR₁ was not an important factor in this model of vascular injury. However, in a rat model of balloon angioplasty, an antibody to PAR₁ reduced neointima formation by ~50% (Takada et al. 1998). Furthermore, RWJ-58259, a PAR₁-selective small-molecule inhibitor, has then been evaluated in a similar rat model of balloon angioplasty and significant inhibition of neointimal thickening after injury was observed (Andrade-Gordon et al. 2001; Damiano et al. 2003). RWJ-58259 was applied locally to the adventitial surface of the injured carotid artery within a polymer gel since this compound is not orally active. Moreover, it has been demonstrated that hirudin, a thrombin inhibitor, prevents angioplasty induced SMC proliferation in rabbits and pigs (Jang et al. 1990a, b; Gertz et al. 1998). These data suggest that PAR₁ plays an important role in restenosis.

12.4.3 Anticancer Therapeutics

A large body of information has accumulated on the involvement of PAR₁ in a variety of malignancies. Upregulation of PAR₁ occurs in metastasis of melanoma cell lines (Tellez and Bar-Eli 2003) and breast cancer cell lines (Even-Ram et al. 1998; Henrikson et al. 1999; Booden et al. 2004); in endometrial carcinomas

(Granovsky-Grisaru et al. 2006), colon adenocarcinomas (Wojtukiewicz et al. 1995), laryngeal carcinomas (Wojtukiewicz et al. 1995), pancreatic carcinomas (Rudroff et al. 1998), and prostate tumors (Black et al. 2007). Thrombin promotes cancer cell survival (Tantivejkul et al. 2005), proliferation (Darmoul et al. 2003, 2004; Tsopanoglou et al. 2004), invasion, and angiogenesis (Henrikson et al. 1999; Even-Ram et al. 2001; Booden et al. 2004; Tsopanoglou and Maragoudakis 2004; Tsopanoglou et al. 2004; Boire et al. 2005). However, there are limited data regarding the effects of a PAR₁ antagonist on tumor growth and metastasis. Two earlier PAR₁ antagonists SCH-79797 and RWJ-56110 were able to block tube formation in the matrigel in vitro system and angiogenesis in the chick embryo angiogenesis model in vivo dose dependently (Zania et al. 2006). Furthermore, the inhibition of angiogenesis by these two compounds may be due to the inhibition of endothelial cell growth by induction of apoptosis (Zania et al. 2006). More recently, two molecular approaches have been used to explore the role of PAR₁ in tumorigenicity and progression (Salah et al. 2007a, b). In one study, PAR₁ antisense RNA attenuated melanoma cell proliferation and invasion in vitro and tumor formation in vivo (Salah et al. 2007b). Another study showed that PAR₁-specific small interfering RNA (siRNA) abolished bombesin-driven prostate cancer cell invasion in a matrigel system (Salah et al. 2007a). Our preliminary results with RWJ-58259 also showed inhibition of tumor cell invasion in a matrigel model (unpublished data). Further research is needed to elucidate the relevance of PAR₁ antagonists in oncology applications.

12.5 Conclusion

Thrombin, an important serine protease in the blood coagulation cascade, can activate certain cell-surface receptors (GPCRs), known as proteinase-activated receptors (PARs), to influence cellular function. Of the four known PARs (designated 1–4), PAR₁, PAR₃, and PAR₄ are sensitive to thrombin. However, PAR₁ has assumed a preeminent position among this group because it is the primary thrombin-responsive receptor in human platelets. Consequently, PAR₁ has been perceived as a high-value molecular target for the discovery and development of novel antiplatelet drugs. Antagonism of PAR₁ can provide benefits over direct thrombin inhibition in that blockade of the thrombin-induced cellular effects mediated by PAR₁ will leave thrombin's role in the coagulation pathway untouched. Thus, it may be possible with PAR₁ antagonists to have effective antiplatelet therapy without bleeding liability.

The road to PAR₁ antagonists that are worthy of being marketed drugs has distinct challenges. One of these is the variability of PAR₁ in the platelets of different species, especially its absence from the platelets of mice, rats and dogs, which are commonly used as preclinical testing models for pharmacology and toxicology. Second, there is the intramolecular nature of the PAR₁ activation mechanism. A PAR₁ antagonist ligand must compete effectively with the native tethered ligand, which has an energetic advantage from an entropy factor. Thus, a designer drug

must have robust affinity, but not from excessive molecular weight. Given this scenario, it is not surprising that researchers have identified very few chemotypes that can interfere with the action of thrombin on human platelets. As a corollary to this aspect, it is thought that PAR₁ antagonist drugs need to have a slow rate of dissociation from the receptor to achieve robust in vivo efficacy. In working with active chemical series, there are no clear structural rules for dialing in this desirable attribute.

Several pharmaceutical companies embarked on studies to find PAR₁ antagonists in the early 1990s. However, most of the compound series did not have what it takes to become viable drug candidates. Peptide-mimetic RWJ-58259 was important because it provided the first in vivo proof-of-concept results in a nonhuman primate antithrombotic model. Unfortunately, this antagonist was not orally bioavailable and had a relatively high molecular weight. Two important compounds emanated from chemical series under study at Eisai and Schering-Plough, and E-5555 and Sch 530348 have advanced into late-stage clinical investigation. Hopefully, these current efforts will lead to oral antiplatelet drugs with a new mechanism of action for the treatment of cardiovascular disorders.

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Chapter 13

Novel Anticoagulant Therapy: Principle and Practice

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Abstract Currently, there are several evidences for the interplay between coagulation and inflammation in the propagation of various disease processes, including venous thromboembolism (VTE) and inflammatory diseases. Major advances in the development of oral anticoagulants are progressing very well, with the goal of developing safe and effective oral anticoagulants that do not require frequent monitoring or dose adjustment along with minimal food/drug interactions. Indirect inhibitors such as low-molecular-weight heparin (LMWH) and the pentasaccharide fondaparinux represent improvements over traditional drugs such as unfractionated heparin for acute treatment of VTE with more targeted anticoagulant approaches, predictable pharmacokinetic profiles, and lack of need for monitoring. Vitamin K antagonist, with its inherent limitations of multiple food and drug interactions and frequent need for monitoring, remains the only oral anticoagulant approved for long-term secondary thromboprophylaxis in VTE. The oral direct thrombin inhibitor ximelagatran was withdrawn from the world market due to safety concerns. Newer anticoagulant drugs such as parenteral pentasaccharides (idraparinux, SSR126517E), oral direct thrombin inhibitors (dabigatran), oral direct factor Xa inhibitors (rivaroxaban, apixaban, YM-150, DU-176b), and tissue factor/factor VIIa complex inhibitors are *tailor-made* to target specific procoagulant complexes and have the potential to greatly expand oral antithrombotic targets for both acute and long-term treatment of venous thromboembolism, acute coronary syndromes, and prevention of stroke in atrial fibrillation patients.

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13.1 Introduction

Inflammation plays a key role in triggering a prothrombotic state via the activation of platelets, coagulation, and vascular insult. Venous thromboembolism (VTE) continues to be a major cause of morbidity and mortality in the western world (Anderson and Spencer 2003). VTE represents 1 in 10 hospital deaths, and postthrombotic syndrome and pulmonary hypertension occur in 10% of deep-vein thrombosis (DVT) and 5% of pulmonary embolism (PE) patients, respectively (Kearon 2003). For more than 50 years, traditional drugs such as unfractionated heparin (UFH) were used parenterally for acute treatment, followed by oral vitamin K antagonists (VKAs) such as warfarin for long-term treatment. These drugs exert their antithrombotic effects by inhibiting multiple steps of the coagulation cascade, with inherent limitations for each drug.

For acute VTE treatment, limitations of UFH include a less than predictable anticoagulant response with the need for frequent monitoring and the potential for severe toxicity, especially heparin-induced thrombocytopenia (HIT) in up to 3% of patients (Warkentin et al. 1995). In the past 15 years, the use of low-molecular-weight heparin (LMWH), and more recently the synthetically derived pentasaccharide fondaparinux has improved our acute management of VTE. A more targeted approach to procoagulant complex inhibition, predictable pharmacodynamic characteristics, and improved safety profiles have enabled complete outpatient treatment of VTE in selected patients without the need for anticoagulant monitoring. Other parenteral drugs, the direct thrombin inhibitors (DTIs) lepirudin and argatroban, have found only limited use in acute VTE treatment, namely in thrombosis associated with HIT.

The optimal long-term treatment of VTE is defined by the limitations of VKAs, the only oral anticoagulants currently approved for use. These limitations include a slow onset of action and need for bridging anticoagulation with a parenteral drug in the acute setting, multiple food and drug interactions, and a narrow therapeutic window, necessitating frequent coagulation monitoring and dose adjustment (Spyropoulos 2006). Additionally, some patient subgroups cannot tolerate VKA, such as pregnant patients requiring anticoagulation, in whom VKA is associated with a risk of teratogenicity (Bates and Ginsberg 1997), or patients in whom VKA is associated with higher risks of recurrent thromboembolism and major bleeding, such as those with active cancer (Hutten et al. 2000; Palareti et al. 2000). In both of these patient groups, emerging data support the use of long-term LMWH (Lee et al. 2003; Bates et al. 2004; Greer and Nelson-Piercy 2005), with limitations of parenteral use.

Improved understanding of molecular mechanisms of coagulation and thrombosis, and its potential to be applied at a clinical level to different patient subgroups, has led to the development of newer antithrombotic drugs for use in VTE treatment. Many of these drugs are orally active, synthetically derived, and target specific procoagulant complexes within the coagulation cascade (Weitz et al. 2004). These drugs can broadly be categorized as interfering with the initiation of coagulation (tissue factor/

Table 13.1 Oral anticoagulants in phase II/III trials

Drugs ^a	Target	Dosing	Adjustment and interactions	Indications
Rivaroxaban (Bayer Schering)	Factor Xa	Once or twice daily	• Avoid or monitor with CrCl <30 mL min⁻¹ • Dose reduction for CrCl 30–49 mL min⁻¹ • Avoid or monitor with strong CYP450 3A4 inhibitors	• Phase III for VTE prophylaxis after joint replacement completed (<i>n</i> = 2,531) • Phase II for secondary prevention after ACS enrolling (<i>n</i> = 1,350) • Phase II for treatment of DVT completed, phase III enrolling (<i>n</i> = 2,900) • Phase III for prevention of stroke in nonvalvular AF enrolling (<i>n</i> = 14,000) • Phase III for treatment of PE enrolling (<i>n</i> = 3,300)
Apixaban (Bristol-Myers Squibb and Pfizer)	Factor Xa	Twice daily	• Avoid or monitor with strong CYP450 3A4 inhibitors	• Phase II trial for treatment of acute DVT completed • Phase III trials for VTE prevention after joint replacement enrolling (<i>n</i> = 7,258) • Phase III trial for prevention of VTE in medical patients (<i>n</i> = 6,524) • Phase III trial for prevention of stroke in nonvalvular AF enrolling (<i>n</i> = 15,000)
PRT054021 (Portola Pharmaceuticals)	Factor Xa	Twice daily	• Undefined yet	• Phase II trial for VTE prophylaxis after joint replacement completed
LY517717 (Eli Lilly)	Factor Xa	Once daily	• Undefined yet	• Phase II trial for VTE prophylaxis after joint replacement completed
Dabigatran (Boehringer Ingelheim)	Factor IIa (thrombin)	Once or Twice daily	• Avoid or monitor with proton pump inhibitors	• Phase III trial VTE prevention after joint replacement completed • Phase III trial for treatment of VTE enrolling (<i>n</i> = 2,554) • Phase III trial for prevention of stroke in nonvalvular AF enrolling (<i>n</i> = 15,000)

^aAdditional oral anticoagulants in preclinical or early clinical developments are not listed

factor VIIa [TF/FVIIa] complex), propagation of coagulation (indirect and direct inhibitors of activated factor X [FXa] or IX [FIXa]), and thrombin activity (DTIs). This chapter will focus on these investigational drugs for VTE treatment, with an emphasis on those undergoing or that have recently completed phase II or III clinical studies (Table 13.1). Additionally, the role of anticoagulants on stroke in atrial

fibrillation patients will be discussed. Atrial fibrillation (AF) is an epidemic, affecting 1–1.5% of the population in the developed world and its significance lies predominantly in a fivefold increased risk of stroke. Strokes associated with AF are usually more severe and confer increased risk of morbidity, mortality, and poor functional outcome. Despite the advances of promising experimental approaches for selected patients with acute stroke, primary prevention with pharmacological agents remains the best approach to reducing the burden of stroke.

13.2 Anticoagulant Targets That Inhibit the Initiation Phase

13.2.1 Tissue Factor/Factor VIIa Complex Inhibitors

The TF/FVIIa complex, as part of the extrinsic system of the coagulation cascade, is considered to be the key system for the initiation of coagulation. In the venous vascular system, it is thought that exposure of TF in orthopedic surgery and in subsets of cancer patients (Dahl 1999; Fernandez et al. 2004) may be responsible for the high risk of VTE in these patient groups, making pharmacological inhibition of the TF/FVIIa complex important (Girard and Nicholson 2001). The function of TF can be blocked by antibodies that prevent the binding of FVIIa to TF, by active-site-inhibited FVIIa, by small molecules or antibodies that block the TF/FVIIa complex function, and by molecules that inhibit the active site of FVIIa in the TF/FVIIa complex after first binding to FXa (Hirsh et al. 2005; Weitz and Bates 2005). Moreover, TF pathway inhibitor (TFPI), a naturally occurring inhibitor, forms a neutralizing complex with TF/FVIIa and FXa (Girard and Nicholson 2001). Thus TFPI, either by upregulation of endogenous TFPI pools or exogenous administration of recombinant TFPI (rTFPI), may be an attractive anticoagulant that acts by blocking the pathological impact of TF/VIIa and Xa in coagulation and beyond (Mousa et al. 2004).

Nematode Anticoagulant Proteins: The hematophagous hookworm, *Ancylostoma caninum*, produces a family of small, disulfide-linked protein anticoagulants (75–84 amino acid residues) referred to as nematode anticoagulant proteins (NAPs) that have been targeted as antithrombotic drugs due to their inhibition of the TF/FVIIa complex. One of these nematode anticoagulant proteins, NAP5, inhibits the amidolytic activity of fXa with $K_i = 43$ pM, and is the most potent natural fXa inhibitor identified thus far. However, unlike NAPc2, NAP5 does not inhibit fVIIa of the fVIIa/TF complex. This is achieved either through binding to FXa alone or, as in the case with NAPc2, in combination with a protein exosite, resulting in potent inhibition of TF/FVIIa. NAPc2 was tested subcutaneously for VTE prophylaxis in a phase I/II clinical trial using mandatory unilateral venography in 293 patients undergoing total knee replacement surgery. The dose of 3 μ g/kg administered within 1 h after surgery was associated with an overall DVT rate of 12.2%, a proximal DVT rate of 1.3%, and a major bleed rate

of 2.3% (Lee et al. 2001). Further clinical trials with NAPC2 are in progress (Moons et al. 2003; Giugliano et al. 2007). Recombinant NAPC2, like other inhibitors of FVIIa/TF including tissue factor pathway inhibitor (TFPI) and active site-blocked FVIIa (ASIS, FFR-rFVIIa, or FVIIai), may have a promising role in the prevention and treatment of venous and arterial thrombosis, as well as potential efficacy in the management of disseminated intravascular coagulopathies, because of their potent and selective inhibition of FVIIa/TF (Moons et al. 2003; Giugliano et al. 2007).

Anti-TF/VIIa: Factor VIIa (FVIIa) is a key serine protease involved in the initiation of the coagulation cascade. It is a glycosylated disulfide-linked heterodimer comprising an amino-terminal gamma-carboxyglutamic acid-rich (Gla) domain and two epidermal growth factor (EGF)-like domains in the light chain, and a chymotrypsin-like serine protease domain in the heavy chain (Edgington et al. 1991). FVIIa requires tissue factor (TF), a membrane-bound protein, as an essential cofactor for maximal activity toward its biological substrates factor X, factor IX, and factor VII (FVII). As such, the TF/FVIIa complex plays an important role in normal physiology as well as in thrombotic diseases such as unstable angina (UA), disseminated intravascular coagulation (DIC), and deep vein thrombosis (DVT). In addition to its function as an initiator of coagulation, TF/FVIIa plays an important role in inflammation and angiogenesis (Lazarus et al. 2004; Arnold et al. 2006). A wide array of strategic approaches to inhibiting the biochemical and biological functions of the TF/FVIIa complex has been pursued. This has been greatly aided from our understanding of the structures for TF, FVII, FVIIa, and the TF-FVIIa complex. These approaches have resulted in inhibitors directed specifically toward either FVIIa or TF. Antagonists include active site inhibited FVIIa, TF mutants, anti-TF antibodies, anti-FVII/FVIIa antibodies, naturally occurring protein inhibitors, peptide exosite inhibitors, and protein and small molecule active site inhibitors. These antagonists can inhibit catalysis directly at the active site as well as impair function by binding to exosites that may interfere with substrate, membrane, or cofactor binding. Different small molecule potent inhibitors of TF/FVIIa reduce thrombus weight in animal models and decrease the level of interleukin-6 (IL-6) in a LPS-stimulated mouse model of endotoxemia (Suleymanov et al. 2003; Lazarus et al. 2004; Buckman et al. 2005; Arnold et al. 2006). Studies were designed to evaluate the antithrombotic efficacy and bleeding propensity of a selective, small-molecule inhibitor of tissue factor/factor VIIa (TF/VIIa) in comparison with small-molecule, selective inhibitors of factor Xa and thrombin in a nonhuman primate model of thrombosis (Suleymanov et al. 2003). Data indicated that TF/VIIa inhibition effectively prevented arterial thrombosis with less impact on bleeding parameters than equivalent doses of factor Xa and thrombin inhibitors (Suleymanov et al. 2003).

TFPI: Data does exist on the anticoagulant effect of TFPI in animals (Bajaj and Bajaj 1997; Mousa et al. 2004) and the possibility that TFPI release may have a role in secondary anticoagulant mechanisms of LMWH in humans (Sandset et al. 1988). rTFPI has only been used in experimental models.

Tissue factor pathway inhibitor (TFPI) is the natural inhibitor of TF coagulant and signaling activities. Studies have shown that TFPI exhibits antiangiogenic and

antimetastatic effects *in vitro* and *in vivo* (Mousa and Mohamed 2004; Amirkhosravi et al. 2007). In animal models of experimental metastasis, both circulating and tumor cell-associated TFPI are shown to significantly reduce tumor cell-induced coagulation activation and lung metastasis (Amirkhosravi et al. 2007). Heparins and heparin derivatives, which induce the release of TFPI from the vascular endothelium, also exhibit antitumor effects, and TFPI may contribute significantly to those effects (Mousa and Mohamed 2004). Indeed, a nonanticoagulant low-molecular-weight heparin with intact TFPI-releasing capacity has been shown to have significant antimetastatic effect in a similar experimental mouse model (Mousa et al. 2006). The evidence supporting the dual inhibitory functions on TF-driven coagulation and signaling strengthens the rationale for considering TFPI as a potential anticancer agent (Bajaj and Bajaj 1997).

Tissue factor pathway inhibitor-2 (TFPI-2), a member of the Kunitz-type serine proteinase inhibitor family, is a structural homologue of TFPI. The expression of TFPI-2 in tumors is inversely related to an increasing degree of malignancy, which may suggest a role for TFPI-2 in the maintenance of tumor stability and inhibition of the growth of neoplasms (Sierko et al. 2007). TFPI-2 inhibits the tissue factor/factor VIIa (TF/VIIa) complex and a wide variety of serine proteinases including plasmin, plasma kallikrein, factor XIa, trypsin, and chymotrypsin. Aberrant methylation of TFPI-2 promoter cytosine–phosphorothioate–guanine (CpG) islands in human cancers and cancer cell lines was widely documented to be responsible for diminished expression of mRNA encoding TFPI-2 and decreased synthesis of TFPI-2 protein during cancer progression (Sierko et al. 2007). TFPI-2 has been shown to induce apoptosis and inhibit angiogenesis, which may contribute significantly to tumor growth inhibition. Restoration of TFPI-2 expression in tumor tissue inhibits invasion, tumor growth, and metastasis, which creates a novel possibility of cancer patient treatment (Sierko et al. 2007).

13.3 Inhibitors of Coagulation Propagation

It is widely accepted that FXa, as part of the prothrombinase complex with FVa, has a central role in clot formation given that it is generated by both the extrinsic and intrinsic pathways of coagulation as they converge into the final common pathway. In addition, within the prothrombinase complex, one molecule of FXa can exponentially generate 138 molecules of thrombin per minute. Theoretically, FXa inhibitors may have advantages over thrombin inhibitors by preventing the activation of coagulation amplification mechanisms as well as thrombin generation in both platelet-rich arterial thrombosis and fibrin-rich venous thrombosis, making FXa a prime target for anticoagulant drug design. However, in animal models of thrombosis, selective FXa inhibition was less potent than direct thrombin inhibition in arterial and venous models of thrombosis, with a greater prolongation of global clotting times, thus refuting the concept that FXa is more effective than direct thrombin activity in controlling thrombin formation (Biemond et al. 1996). Finally, the

theoretical possibility that selective inhibition of FXa upstream may result in a safer bleeding profile exists; in the absence of thrombin activity inhibition, small amounts of thrombin would escape neutralization and facilitate hemostasis.

Inhibitors of FXa include both indirect (antithrombin [AT]-mediated) and direct AT-independent selective inhibitors. Other possible targets of coagulation propagation, via the prothrombinase complex or other routes, include FIXa inhibitors, FVIIIa and FVa inhibitors, activated protein C, or soluble thrombomodulin.

13.3.1 Indirect Factor Xa Inhibitors

The synthetically derived pentasaccharides fondaparinux and idraparinux represent the most developed selective indirect FXa inhibitors. They exert their action by high-affinity binding and activation of AT, which then inhibits free FXa. Fondaparinux has the pentasaccharide sequence found in heparins and selectively binds to and induces a conformational change in AT, increasing the anti-Xa activity of AT nearly 300-fold in a catalytic fashion. Fondaparinux has a linear pharmacokinetic profile and predictable anticoagulant response with a plasma half-life of approximately 18 h and >95% bioavailability after intravenous or subcutaneous injection, allowing nonmonitored once-daily subcutaneous dosing. In addition, it does not bind to platelet factor 4, and has not been associated with drug-induced thrombocytopenia. Fondaparinux is currently approved for acute treatment of DVT and PE based on the recently completed MATISSE studies in VTE (Buller et al. 2003, 2004). As such, fondaparinux represents the first drug of a class of selective indirect FXa inhibitors providing proof of concept that FXa inhibitors can treat thrombosis in the acute stage as efficaciously as drugs with established AT activity. Fondaparinux also represents the first of a new class of antithrombotic drugs designed specifically to inhibit a single target or procoagulant complex in the coagulation cascade.

Idraparinux (Sanofi-Aventis): Idraparinux is a hypermethylated, long-acting pentasaccharide, allowing once-weekly dosing. Idraparinux sodium is a second-generation pentasaccharide with sulfated side chains resulting in a 30-fold higher binding affinity to AT compared with fondaparinux, and a 120-h elimination half-life, allowing once-weekly administration (Herbert et al. 1998). It has similar advantages to fondaparinux such as 100% bioavailability after parenteral administration, linear pharmacokinetics, predictable anticoagulant response with no need for monitoring, lack of induction of platelet aggregation, lack of effects of platelet factor 4, and no evidence of induction of thrombocytopenia. The major drawback, as for fondaparinux, has been the lack of an antidote, although its importance in clinical practice is controversial. However, it should be noted that biotinylated idraparinux, discussed in detail later, does have an antidote.

The PERSIST study, a randomized, phase II, dose-ranging study, compared idraparinux with warfarin for a 12-week treatment of DVT (after initial enoxaparin demonstrated efficacy with all idraparinux doses that were similar to warfarin). No clear dose-response relationship for efficacy was shown but a significant dose-response

relationship for major bleeding was shown with idraparinux (Reiter et al. 2003). The large phase III trial comparing the efficacy and safety of idraparinux with a heparin or fondaparinux and dose-adjusted warfarin in both acute and long-term treatment of DVT and PE has recently been completed (Reiter et al. 2003).

SSR126517E (Biotinylated Idraparinux): This synthetic pentasaccharide being developed by Sanofi-Aventis has antithrombotic properties resulting from AT mediation of FXa activity. It is identical to idraparinux with the exception of a biotin moiety being covalently affixed through a linker to the pentasaccharide structure so that anti-FXa activity may be neutralized in vivo by a specific protein, avidin. In vitro studies reveal that SSR126517E (biotinylated idraparinux) shows high-affinity binding (dissociation constant $[K_d] = 10^{-9} \text{ mol L}^{-1}$) to human AT with a concentration-dependent inhibition of FXa. It does not inhibit platelet aggregation or cross-react with antibodies from sera of patients with HIT. In phase I studies, the median time to reach the maximum concentration was 4 h with an absolute bioavailability of 100%, and a half-life of approximately 200 h. Exposure of this compound to avidin revealed a rapid decrease of anti-Xa activity and no serious adverse events (van Gogh Investigators et al. 2007).

13.3.2 Selective Direct Factor Xa inhibitors

Advantages of direct FXa inhibitors include the absence of intermediary molecules such as AT that may potentially result in inconsistent anticoagulation, especially during acute or inflammatory states. DX-9065a was the first of a class of small, synthesized, selective direct FXa inhibitors to undergo phase II clinical trials in arterial thrombosis (Alexander et al. 2005). Efforts to develop orally available selective FXa inhibitors for VTE and prevention of stroke in AF patients are underway (Table 13.1).

Razaxaban: Razaxaban (BMS-561389), developed by Bristol-Myers Squibb (formerly Dupont), represented the first of a new class of synthetically derived, small molecule, oral direct FXa inhibitors not requiring anticoagulant monitoring. It was well tolerated and well absorbed in phase I trials involving young and elderly volunteers with only nuisance bleeding reported. Additionally, FXa inhibition and dose-dependent anticoagulation were noted. Razaxaban was studied as proof of principle for DVT prevention in patients undergoing total knee replacement. In the phase IIb trials, Razaxaban 25, 50, 75, or 100 mg twice daily (bid) initiated 8 h after surgery was compared with enoxaparin 30 mg twice daily initiated 12–24 h after surgery. The study revealed efficacy but an unacceptable risk/benefit profile at higher doses (Lassen et al. 2007). It was discontinued for further development in March 2005 due to an oral FXa inhibitor under development by the same company with a more favorable safety profile.

Apixaban: Apixaban (formerly BMS-562247 or DPC-AG0023) is another orally active, small-molecule direct FXa inhibitor being developed by Bristol-Myers Squibb with a more favorable safety profile than Razaxaban. It is a highly potent

inhibitor of human FXa, with an inhibition constant (K_i) of 0.08 ± 0.01 nM, and 87% is bound to serum proteins. It has consistent oral absorption and linear pharmacokinetics with maximal plasma concentration achieved within 3 h, and an effective half-life of 9 h for twice-daily administration and 14 h for once-daily administration. It is eliminated via both hepatic and renal routes. Apixaban had only modest effects on two traditional markers of anticoagulation, International Normalized Ratio (INR) and activated partial thromboplastin time (aPTT). Phase I clinical studies revealed mild bleeding and a prolongation of the bleeding time, without evidence of high elevation of transaminases (alanine transaminase or aspartate transaminase ≥ 5 times the upper limit of normal levels). Its safety profile remains to be determined in phase II/III trials, and it is presently undergoing phase II clinical studies in elective total knee replacement surgery and acute DVT treatment.

The efficacy and safety of apixaban as thromboprophylaxis in patients following total knee replacement was determined (Lassen et al. 2007). Apixaban in doses of 2.5 mg (bid) or 5 mg (qd) has a promising benefit–risk profile compared with the current standards of care following TKR (Lassen et al. 2007).

Rivaroxaban (Bayer HealthCare AG and J&J/Scios, Inc.): Rivaroxaban (formerly BAY 59–7939) is a small molecule, selective oral direct FXa inhibitor developed by Bayer for the prevention and treatment of thrombosis. Oral rivaroxaban may be given in fixed once-daily doses, with the potential for no coagulation monitoring. These properties, along with results from preclinical and clinical studies, suggest that rivaroxaban may have advantages over current treatments. Studies in arterial and venous animal models demonstrated that rivaroxaban has potent antithrombotic effects, without prolonging bleeding times. In healthy subjects, rivaroxaban was well tolerated with a predictable pharmacological profile and a low propensity for clinically relevant drug–drug interactions. In preclinical studies, endogenously generated FXa was inhibited with an IC_{50} of 21 ± 1 nM (Perzborn et al. 2005). The antithrombotic effect was demonstrated in different thrombosis models at 0.6–10 mg kg^{-1} given orally, depending on models and species. In dogs, the bioavailability was 60–86%.

In phase I studies, rivaroxaban was rapidly absorbed (maximum concentration [C_{max}] reached after 30 min) and well tolerated (up to 80 mg after a single dose in healthy people) (Kubitza et al. 2005). Elimination occurred with terminal half-lives of 4.86–9.15 h (steady state). Prothrombin time (PT), aPTT, and HepTest were prolonged dose-dependent, and there was no influence on bleeding time. In elderly men and women (>60 years), mean area under the concentration–time curve and C_{max} tended to be about 20% higher. There were no drug–drug interactions or induction of major cytochrome P450 (CYP) isoforms, with the exception of strong CYP3A4 inhibitors, and no QTc prolongation was observed.

Four large dose-ranging studies (ODIXa-HIP, ODIXa-HIP2, ODIXa-KNEE, and ODIXa-OD.HIP) have been completed, exploring a 12-fold rivaroxaban dose range from 2.5 to 30 mg (bid), and 5–40 mg once daily (qd) for VTE prevention in major orthopedic surgery (Turpie et al. 2005; Eriksson et al. 2006). The open-label phase IIa study ODIXa-HIP using mandatory venography confirmed proof of

principle of rivaroxaban for this indication. Studies have confirmed the efficacy and safety of rivaroxaban compared with enoxaparin under double-blind, double-dummy conditions for VTE prevention in patients undergoing orthopedic surgery (Eriksson et al. 2006). Phase II studies of rivaroxaban for the prevention of venous thromboembolism (VTE) after major orthopedic surgery support these findings. The results also suggested that a total daily dose range of 5–20-mg rivaroxaban had similar efficacy and safety to enoxaparin, and that 10-mg rivaroxaban once daily was the optimal dose (Laux et al. 2007).

The drug development plan for rivaroxaban is aggressive, with simultaneous investigative programs spanning multiple indications rather than a sequential approach. At present, over 24,000 patients (12,000 are predominantly postoperative orthopedic patients) have been evaluated in completed phase II and III trials of rivaroxaban for thromboprophylaxis and treatment of DVT. By the time all the currently enrolling trials have concluded, more than 50,000 patients will have been evaluated in all randomized controlled trials of rivaroxaban. The advantages of rivaroxaban include the potential for once-daily dosing for all indications, no required dose adjustment for body weight, no known interactions with common cardiovascular medications, a relatively safe pharmacodynamic profile with respect to bleeding risk and hepatotoxicity, no clinically significant interaction with aspirin, and the ability to bridge with low-molecular-weight heparin when necessary. On the other hand, rivaroxaban is partially renally cleared and will require dose adjustment in those with grade III chronic kidney disease, and is not being studied in patients with a creatinine clearance less than 30 mL min⁻¹. Additionally, since rivaroxaban's metabolism is affected by potent cytochrome P450 3A4 inhibitors, such as ketoconazole, clarithromycin, and protease inhibitors, its use will be restricted in some special populations. Nonetheless, after extensive phase II data, and now emerging phase III trial data, it appears that rivaroxaban is effective in preventing and treating VTE with a bleeding risk comparable with other anticoagulants. The results of randomized trials evaluating rivaroxaban for the prevention of stroke and non-CNS embolism in AF and secondary prevention of acute coronary syndromes are currently ongoing.

While there are several other oral anticoagulants in development, none have been evaluated as extensively or in as many patients as rivaroxaban. Rivaroxaban also has the advantage that it can be dosed once a day, which has been shown to improve patient compliance and outcomes (Claxton et al. 2001; Richter et al. 2003). Despite once daily dosing, rivaroxaban has a half-life that is considerably shorter than other oral FXa inhibitors, which is an advantage in the event of bleeding or an urgent need to discontinue anticoagulation. Unlike the direct thrombin inhibitor dabigatran, rivaroxaban has very good bioavailability and has low potential for drug–drug interactions, including medications that alter gastric pH, which are taken chronically by 3% of the US population (Jacobson et al. 2003). Perhaps more importantly, rivaroxaban has been shown to have no effect on platelet aggregation and its pharmacokinetic profile is unaffected by aspirin and other notable cardiovascular medications such as digoxin. Finally, after clinical investigation in thousands of patients, rivaroxaban appears to have no significant hepatotoxicity and a bleeding risk comparable with other conventional anticoagulants.

Rivaroxaban has potential use in several clinical indications and disease states including venous thromboembolism prophylaxis, long-term treatment of DVT, PE, and the prevention of stroke and non-CNS embolism in patients with atrial fibrillation, and potentially acute coronary syndromes. Therefore, in order to understand the efficacy and safety for such a therapeutic across such a wide range of patient populations, several large simultaneous studies evaluating rivaroxaban in both venous and arterial thromboembolism are under way. The therapeutic may have its greatest impact in providing a much-needed and attractive alternative to warfarin. While formal cost-effective analyses are not yet available, avoidance of the intensive, costly, and frequent monitoring required with VKAs as well as a potential reduction in adverse vascular events precipitated by the narrow therapeutic window of VKAs should result in a significant improvement in quality-of-life and cost savings. While further data (especially large phase III trials) and caution are required, there is reason for optimism. Rivaroxaban may very well be the long-awaited alternative to warfarin.

YM-150: Astellas (formerly Yamanouchi) has developed YM-150, an oral selective FXa inhibitor for DVT prevention. The compound reveals an immediate antithrombotic effect after oral administration, with a dose-dependent response and prolongation of PT. There was also no significant food interaction effect noted. In a phase II dose-escalation study in patients undergoing elective primary hip replacement surgery, YM-150 [3, 10, 30, or 60-mg PO (qd)] given 6–10 h after surgery for 7–10 days was compared with enoxaparin 40-mg SQ (qd) given 12 h before surgery (Eriksson et al. 2005b). There were no major bleeds. The median incidence of VTE ranged from 52% in the 3-mg group to 19% in the 60-mg group. Overall, the drug appeared to be safe and well tolerated. A dose escalation study of YM150 in the prevention of venous thromboembolism in elective primary hip replacement surgery was carried out (Eriksson et al. 2007b). YM150, 10–60 mg daily, starting 6–10 h after primary hip replacement, was shown to be safe, well tolerated, and effective.

DU-176b: Daiichi Sankyo is developing DU-176b, an oral FXa inhibitor for the potential treatment of thrombotic disorders. Preclinical data in mice models revealed potent antithrombotic effects in both AT-positive and AT-deficient mice (Fukuda et al. 2005). In rat models, DU-176b at doses of 0.05–1.25 mg kg⁻¹ h⁻¹ resulted in prevention of both arterial and venous thrombosis.

DU-1766 in a single 60-mg dose of DU-176b was given to healthy males. It inhibited FXa activity, reduced thrombin generation, prolonged PT, aPTT, and INR, and reduced venous thrombosis by 28% and arterial thrombosis by 26% in a Badimon chamber. Further studies are planned.

LY-517717: LY-517717, an indol-6-yl-carbonyl derivative, is the lead in a series of oral selective FXa inhibitors being developed by Eli Lilly as part of a research collaboration with Protherics for the potential treatment of thromboembolic diseases. It is a 1,000-fold more selective FXa inhibitor than other serine proteases, with a Ki of 5 nM. Its oral bioavailability is approximately 25–82%, with a plasma half-life of 7–10 h. In a rat atrio-ventricular shunt model, the compound had a median effective dose of 5–10 mg kg⁻¹ PO, and absorption in dogs suggests no bleeding issues. In a phase I study, LY-517717 was found to be well tolerated and suitable for once-daily administration. In a dose-escalating study, 511 patients undergoing hip or knee replacement surgery

were randomized to receive one of six oral doses of LY-517717 (25, 50, 75, 100, 125, or 150 mg), or enoxaparin 40-mg SQ daily, started preoperatively, for 6–10 doses. The 100, 125, and 150-mg doses of LY-517717 were found to be noninferior to enoxaparin in the incidence of symptomatic or venographically proven DVT or PE (Agnelli et al. 2005). The compound produced a dose-dependent prolongation of PT and was well tolerated, with no differences in bleeding risk compared with enoxaparin (Agnelli et al. 2005).

13.3.3 Selective Direct Factor IXa Inhibitors

Although developments of direct FIXa inhibitors are in an earlier phase than direct FXa inhibitors, the theoretical advantages should be similar. TTP 889 manufactured by Trans Tech Pharma is an oral, direct factor IXa inhibitor with a half-life of 20 h, enabling once-daily dosing. FIXIT is a phase II proof of principle study for VTE prevention in hip fracture surgery that recently completed enrollment of 206 patients who received standard in-hospital thromboprophylaxis. Efficacy and safety are compared between patients who are randomized to receive TTP 889 vs. placebo for up to 3 weeks postdischarge (Rothlein et al. 2005).

13.3.4 Factor XIa Inhibitors

Factor XIa inhibitors are at the preclinical levels. The effect of inhibiting activated blood coagulation factor XIa was determined in rat models of thrombosis and hemostasis using BMS-262084, an irreversible and selective small-molecule inhibitor of factor XIa with an IC(50) of 2.8 nM against human factor XIa (Schumacher et al. 2007). BMS-262084 doubled the activated thromboplastin time in human and rat plasma at 0.14 and 2.2 μM , respectively. Consistent with factor XIa inhibition, the prothrombin time was unaffected at up to 100 μM . BMS-262084 administered as an intravenous loading plus sustaining infusion was effective against ferric chloride (FeCl_2)-induced thrombosis in both the vena cava and carotid artery. In contrast, doses of up to 24 mg kg^{-1} + 24 mg $\text{kg}^{-1} \text{ h}^{-1}$ had no effect on either tissue factor-induced venous thrombosis or the ex vivo prothrombin time. Doses of up to 24 mg kg^{-1} + 24 mg $\text{kg}^{-1} \text{ h}^{-1}$ also did not significantly prolong bleeding time provoked by either puncture of small mesenteric blood vessels, template incision of the renal cortex, or cuticle incision. These results demonstrate that pharmacologic inhibition of factor XIa achieves antithrombotic efficacy with minimal effects on provoked bleeding (Schumacher et al. 2007).

13.4 Inhibitors of Thrombin Activity

Thrombin is the central serine protease in hemostasis with mechanisms of action affecting coagulation, platelet activation, fibrinolysis, and vascular cell biology. In addition to its major role in fibrin formation and activation of FXIII, which cross-

links fibrin, it is essential for feedback activation of other coagulation factors such as FV, FVIII, and FIX (Lundblad et al. 2004). Thrombin also has a role in platelet activation and subsequent aggregation (Agnelli et al. 2005). Lastly, thrombin acts as an anticoagulant by binding to thrombomodulin, which converts protein C to its active form, inactivating FVa and FVIIIa. Also, thrombin has a regulatory role in coagulation by downregulating fibrinolysis when thrombomodulin-bound thrombin activates thrombin-activatable fibrinolysis inhibitor (TAFI). The theoretical considerations that thrombin inhibitors may be more effective than FXa inhibitors in arterial thromboembolic disease (where thrombin has a key role in platelet activation) and less effective in VTE have not been borne out by clinical data. Given its central role in the coagulation cascade, inhibitors of thrombin activity – whether mediated by AT or acting directly on the active site – represent an important class of anticoagulant drug in our armamentarium.

13.4.1 Indirect Thrombin Inhibitors: SNAC/Heparin

SNAC (sodium *N*-[8(2-hydroxybenzoyl)amino]caprylate) developed by Emisphere Technologies enabled macromolecule delivery of the large negatively charged and poorly absorbed heparin molecule via a noncovalent complex with heparin that allowed passive, transcellular absorption. SNAC itself has no pharmacological activity. Phase I studies revealed that in doses up to 10.5-g SNAC per 150,000-U heparin, the compound was well tolerated with nausea as the only significant adverse event observed. There were dose-dependent increases in the aPTT and anti-FXa levels, suggesting that both AT-mediated thrombin and FXa inhibition have a role in its anticoagulant effects (Baughman et al. 1998). There was an apparent food and diurnal effect observed but no age effect. The PROTECT study was a large phase III study in 2,264 hip-replacement patients with two treatment arms of SNAC (low-dose SNAC and high-dose SNAC) for 30 days vs. 10 days of enoxaparin 30 mg subcutaneously (SQ) every 12 h (Hull et al. 2001).

Mandatory venography on days 27–30 revealed an overall VTE rate of 31.8% in the low-dose SNAC group, 29.7% in the high-dose group, and 26.1% in the enoxaparin group. The rates of proximal DVT/PE were 18.6% in the low-dose group and 13.8% in the high-dose group, both of which were significantly higher than in the enoxaparin group (12.7%, $p = 0.013$ and $p = 0.045$, respectively). There was overall poor compliance in 22.1% of the patients on the low-dose regimen and in 31.4% of the patients on the high-dose regimen, suggesting that substance compliance may be the key factor in why the phase III study with SNAC/heparin did not attain proof of principle for its use in VTE prevention as an indirect FIIa/Xa inhibitor. However, further improvements were made in formulating heparin in solid dosage form, which is under clinical investigation (Kim et al. 2007; Mousa et al. 2007). An orally administrable chemical conjugate of heparin and hydrophobic deoxycholic acid (DOCA), which we refer to as LHD was developed. LHD was preformulated with dimethyl sulfoxide (DMSO) as a solubilizer to further improve its oral bioavailability (9.1% in monkeys). LHD was found to be absorbed mainly in the jejunum and

ileum of the small intestine, although it is in the ileum that the absorption is most notable. From the mechanism studies of LHD absorption using Caco-2 cell monolayers for mimicking the intestine, we found that LHD highly permeated by passive diffusion through the transcellular route and its permeation was partially affected by bile acid transporters. This study demonstrates the feasibility of chemically modified heparin for long-term oral administration as an effective therapy for venous thromboembolism in clinical trials (Kim et al. 2007). Recent clinical study determined for the first time the true PK for injectable vs. oral heparin (Mousa et al. 2007).

13.4.2 Direct Thrombin Inhibitors

The development of DTIs was driven by three major factors: the increasing recognition of immune thrombocytopenia as a potentially severe complication of heparin use (Kelton 2005), the notion that heparin-AT inhibition of thrombin produces only weak inhibition of cell-surface- or clot-bound thrombin and that this bound thrombin is active and can be released during fibrinolysis (Weitz et al. 1990), and the nonspecific binding properties of heparin that necessitate frequent monitoring. Hence, non-AT-based thrombin inhibitors that have an improved safety profile over heparin, the ability to inhibit surface- or clot-bound thrombin, and predictable dose responses may have clinical advantages. Oral formulations of these drugs would also represent a major advantage. DTIs may also be ideal drugs for HIT treatment due to the generation of large amounts of thrombin associated with this condition. Lastly, a theoretical concern of DTIs was the inhibition of the anticoagulant properties of thrombin, namely inhibition of the thrombin–thrombomodulin-mediated negative feedback mechanism of the protein C system, with the possibility of rebound hypercoagulability (Mattsson et al. 2001).

Four parenteral DTIs have emerged: lepirudin, bivalirudin, argatroban, and melagatran, with the first three having been approved for clinical use. Lepirudin is a naturally occurring bivalent DTI indicated for thromboprophylaxis of HIT. Argatroban is the prototype noncovalent, reversible, small-molecule DTI indicated for thromboprophylaxis or treatment of HIT. Melagatran is the active form of the oral, prodrug, small-molecule DTI ximelagatran, discussed later. All these drugs have limitations in terms of parenteral use, limited indications, need for frequent monitoring, and high cost.

13.4.3 Selective Oral Direct Thrombin Inhibitors: Ximelagatran

Ximelagatran, developed by AstraZeneca, represented the first of a new class of orally active, small-molecule DTIs to reach late-stage development and limited clinical indications for VTE prevention. Ximelagatran was the hydrophilic prodrug

converted by a cytochrome-P450-independent liver enzyme system to its active form melagatran, with approximately 20% bioavailability and a half-life of 4–5 h in patients. It was administered twice daily and did not require anticoagulant monitoring or dose adjustment. Ximelagatran was studied extensively in a large phase III program for VTE prevention and treatment and was found to be either superior or equivalent to warfarin in terms of efficacy (Francis et al. 2002, 2003; Eriksson et al. 2003a, b; Schulman et al. 2003; Fiessinger et al. 2005). However, overall initial long-term data with ximelagatran revealed liver enzyme elevations of about 6%, and based upon this and other considerations the US Food and Drug Administration did not approve its application. It was, however, approved in other countries for short-term, post-orthopedic thromboprophylaxis. In February 2006, AstraZeneca withdrew ximelagatran from the world market due to continuing concerns of severe liver toxicity with long-term use.

Dabigatran: Dabigatran etexilate is another small-molecule, orally active, prodrug DTI developed by Boehringer Ingelheim that has reached late-stage clinical development. It is rapidly absorbed and converted to the active form, dabigatran. It has linear characteristics between concentration and global coagulation parameters, including thrombin clotting time, INR, and ecarin clotting time. Dabigatran has a K_i of 4.5 ± 0.2 nmol L⁻¹, a peak plasma concentration at 2-h postdose, and a half-life of approximately 14–17 h after multiple dose administration (Stangier et al. 2001). It is mainly metabolized (80–85%) via renal excretion.

The BISTRO II study was a multicenter, parallel group, double-blind, dose-finding study for VTE prevention in 1,949 patients undergoing total hip or knee replacement (Eriksson et al. 2005a). Patients were randomized to receive dabigatran 50 mg (bid), 150 mg (bid), 225 mg (bid), or 300 mg (qd), initiated 14 h after surgery. The comparator was enoxaparin 40 mg (qd) initiated 12 h prior to surgery. A significant dose-dependent decrease in DVT occurred with increasing doses of dabigatran ($p > 0.001$), and, compared with enoxaparin, DVT was significantly lower in patients receiving dabigatran 150 mg (bid) (odds ratio [OR] 0.47, $p = 0.0007$), 300 mg (qd) (OR 0.61, $p = 0.02$), and 225 mg (bid) (OR 0.47, $p = 0.0007$). Compared with enoxaparin, major bleeding was lower with dabigatran 50 mg (bid) (0.3% vs. 2.0%, $p = 0.047$), but elevated at higher doses, with trends almost reaching statistical significance in those receiving the 300-mg dabigatran dose (4.7%, $p = 0.051$). In terms of adverse events, the incidence of elevated alanine aminotransferase $>3 \times$ ULN was lower in the dabigatran groups (1.5–3.1%) than in the enoxaparin group (7.4%). There were no cases of clinically relevant thrombocytopenia. The authors concluded that dabigatran started in the early postoperative period was effective and safe across a wide range of doses. Additionally, the frequency and extent of severe hepatic abnormalities in this study are lower than those observed with ximelagatran. Dabigatran is currently undergoing extensive phase III evaluation in VTE prevention, treatment, and secondary thromboprophylaxis with its RE-VOLUTION program. A randomized, double-blind, noninferiority trial was conducted comparing dabigatran etexilate vs. enoxaparin for prevention of venous thromboembolism after total hip replacement (Eriksson et al. 2007a). In this double-blind randomized study,

3,494 patients undergoing total hip replacement were subjected to treatment for 28–35 days with dabigatran etexilate 220 mg ($n = 1,157$) or 150 mg ($n = 1,174$) once daily, starting with a half-dose 1–4 h after surgery, or subcutaneous enoxaparin 40 mg once daily ($n = 1,162$), starting the evening before surgery. The primary efficacy outcome was the composite of total venous thromboembolism (venographic or symptomatic) and death from all causes during treatment. On the basis of the absolute difference in rates of venous thromboembolism with enoxaparin vs. placebo, the noninferiority margin for the difference in rates of thromboembolism was defined as 7.7%. Both doses of dabigatran were noninferior as compared with enoxaparin. There was no significant difference in major bleeding rates with either dose of dabigatran etexilate compared with enoxaparin (Eriksson et al. 2005a). The frequency of increases in liver enzyme concentrations and of acute coronary events during the study did not differ significantly between the groups. The study concluded that oral dabigatran etexilate was as effective as enoxaparin in reducing the risk of venous thromboembolism after total hip replacement surgery, with a similar safety profile (Eriksson et al. 2007a).

TGN-167: TGN-167 (TRI-50c-04) is an oral thrombin inhibitor being developed by Trigen Holdings for the potential treatment of thrombosis. A controlled-release formulation of the drug is also being developed with Eurand for long-term treatment of thrombosis. The compound produces a marked increase in thrombin clotting time, with minimal effects on aPTT. A double-blind, phase I, dose-escalation study with 20 volunteers showed the drug to be well tolerated, with no significant adverse events reported (Coombe et al. 2005). At 600 mg, all dosed subjects achieved in vitro effective anticoagulant activity. Trigen is planning to continue TGN-167 into phase II studies.

13.5 Expert Opinion and Conclusions

The antithrombotic management of VTE will undergo significant changes in the next 5–10 years. The limitations of existing parenteral and oral anticoagulants have led to the development of newer agents designed to target specific procoagulant complexes in the coagulation pathway inhibiting coagulation initiation, coagulation propagation, or thrombin activity. During acute treatment of VTE, with respect to efficacy, newer antithrombotic agents must exhibit at least noninferiority in a methodologically sound design with respect to the existing parenteral agent of choice – LMWH – and the newer emerging agent fondaparinux. This especially holds true in high-risk venous thromboses such as iliofemoral VTE, PE, or VTE associated with cancer. For long-term VTE treatment, there is a need to improve upon the existing oral anticoagulants – the VKAs. The target-selective oral agents must exhibit an improved safety profile (especially as pertains to major or clinically significant bleeding), ease-of-use, and tolerability when compared with the VKAs. If successful, these emerging oral anticoagulant drugs have the potential to negate

the traditional distinction of acute vs. long-term treatment of VTE, as they may be used throughout the spectrum of disease, without the need for overlap with parenteral therapies (Fukuda et al. 2005). Lastly, any new long-term anticoagulant must be safely tolerated in combination with antiplatelet agents, as increasingly an aging population will be prone to arterial as well as venous thromboembolic disease. Cost considerations are also important, especially from a populational perspective.

All these newer agents should, in theory, fulfill the requirements of an ideal anticoagulant: a rapid onset with predictable response characteristics, predictable pharmacokinetics, pharmacodynamics with low plasma protein binding, lack of need for monitoring, a half-life that provides both safety and ease of use (especially during temporary withdrawal), lack of food or drug interactions, an excellent safety profile (especially with respect to immune-mediated thrombocytopenia, hepatotoxicity, and potential for thrombotic rebound phenomenon), and reversibility or availability of an antidote. In addition, oral agents with predictable intestinal absorption/bioavailability used in a simple, fixed-dose once or twice daily regimen and with the ability to have compliance monitored would present further advantages. At this time, drugs at the most advanced stage of development with respect to VTE management include the parenteral indirect FXa inhibitor idraparinux and biotinylated idraparinux, the oral DTI dabigatran, and the oral selective direct FXa inhibitors such as rivaroxaban and apixaban. Whether there are inherent advantages in blocking initial thrombin formation via the prothrombinase complex early in the coagulation system or blocking thrombin directly and preventing feedback amplification is still a matter of debate, as is the notion of whether there is any clinically meaningful effect of small-molecule DTIs that target both clot-bound and free thrombin. Long-term clinical data with respect to efficacy of anti-Xa inhibitors will be available shortly, while long-term data are available on the efficacy of direct thrombin inhibition. The lessons from ximelagatran reveal the importance of long-term safety data in different patient populations. Ximelagatran had shown significant potential as a possible replacement to warfarin therapy, but has been withdrawn because of potential liver toxicity. Its contrast, dabigatran, appears to have a better safety profile and has recently entered a phase III randomized clinical trial in AF. Oral direct factor Xa inhibitors (rivaroxaban, apixaban, and others) may prove to be more potent and safe. Selective inhibitors of specific coagulation factors involved in the initiation and propagation of the coagulation cascade (factor IXa, factor VIIa, circulating tissue factor) are at an early stage of development. Additional new agents in clinical development include nematode anticoagulant peptide (NAPc2), protein C derivatives, and soluble thrombomodulin.

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Index

A

- Activated protein C (APC)
 - adhesion molecule expression, 67–68
 - antithrombotic effect
 - acute thrombus formation, 13–14
 - enoxaparin and WE doses, 15
 - thrombin analog WE infusion, 14–15
 - apoptosis, 68–69
 - barrier integrity protection, 67
 - inflammation, 65
 - signaling, PAR, 65–66
 - surface retention, 75
- Activator protein-2 α (AP-2 α)
 - and PAR 1, 182
- Alzheimer's disease
 - apolipoprotein E-4 (apoE-4), 146
 - A β -induced toxicity, 145
 - inflammatory proteins, 145
 - microtubule-associated protein tau, 144–145
- Amyloid precursor protein (APP), 145
- Angiogenesis, 163, 164
 - blood vessel formation
 - endothelial progenitor cells (EPCs), 94–95
 - vascular endothelial growth factor-A, 95
 - cancer therapy target and ischemic disorders, 96–97
 - coagulation system
 - activated protein C (APC) and protein C inhibitor (PCI), 98
 - thrombin, 98–99
 - tissue factor (TF), 97
 - tissue factor pathway inhibitor (TFPI), 97–98
 - ECM and integrin interaction, 96
 - negative regulation
 - thrombospondin, 95
 - vasohibin, 96
 - thrombin
 - arterial protection, 104–105
 - endothelial cell survival, 104
 - PAR 1 activation, 103–104
 - vascular network formation, 105
 - thrombin-induced angiogenesis
 - coagulation-dependent pathways, 99–102
 - coagulation-independent mechanisms, 102–103
 - fibrin, 105
 - PAR 1 antagonists, 105–106
 - TP508, 121–122
- Anti-coagulant protease activated protein C, 56
- Antithrombotic agents, 224
- Anti-thrombotic therapy, cancer patients
 - anti-thrombotic and cancer survival, 197–198
 - clinical challenges and epidemiology, 192–194
 - secondary recurrence prevention, 196–197
 - thromboprophylaxis
 - primary surgical thromboprophylaxis, 195–196
 - UFH and LMWHs, 194, 195
 - VTE prevention, 196
 - tissue factor and cancer procoagulant, 191
 - Virchow's triad in, 190
 - VTE treatment, 196
- Apixaban, 244–245
- Apolipoprotein E-4 (apoE-4), 146
- Apoptosis, APC, 68–69
- Argatroban, 165
- Astrocytes
 - cell differentiation and proliferation, 136, 137
 - cell morphology of, 135
 - neuroinflammation, 139
 - synaptic plasticity, 137
- Atrial fibrillation (AF), 237

B

Bombesin, 182, 183

Brain, thrombin and thrombin receptors role
Alzheimer's disease

apolipoprotein E-4 (apoE-4), 146

A β -induced toxicity, 145

inflammatory proteins, 145

microtubule-associated protein tau,
144, 145

HIV infection, 148–149

inhibitors role, brain injury, 134, 135

multiple sclerosis (MS), 147–148

NADPH oxidase, 138, 145

neural development and plasticity

astrocytes and neurons cell

morphological changes, 135

cell differentiation and proliferation,
136, 137

learning and memory, 137

PAR₁-mediated signal transductions
in, 136

RhoA activation, 135, 136

neural prothrombin, 133, 134

neuroinflammation

astrocytes, 139

GRO/CINC-1 release, 139, 140

microglial activation, 138

and Parkinson's disease, 146–147

physiological and pathological roles,
149, 150

protease-activated receptors

(PARs), 134

and stroke

apoptosis, 143

glutamate and, 143

ischemic brain, 140

PAR₁ activation, 142, 143

TNF- α and iron, 142

TPC and brain edema, 141

C

Cancer procoagulant (CP), 191

β -Catenin, *hPar1*, 178–179

Cell-type-specific thrombin receptor signaling

endothelial cells, 51–52

fibroblasts and smooth muscle cells, 52

platelets, 51

sensory neurons and glial cells, 52

Clathrin-mediated endocytosis, PAR

adaptor protein complex-2 (AP-2), 54–55

arrestin-independent internalization, 54

ubiquitination, 55

Coagulation

angiogenesis

activated protein C (APC) and protein
C inhibitor (PCI), 98

thrombin, 98–99

tissue factor (TF), 97

tissue factor pathway inhibitor (TFPI),
97–98

cascade reaction, 82–83

extrinsic pathway

Factor VII, 85–86

tissue factor, 83, 85

FX protein and nonenzymatic FV

cofactor, 86

intrinsic pathway, 83

propagation inhibitors, VTE

factor XIa inhibitors, 248

indirect factor Xa inhibitors, 243–244

selective direct factor IXa inhibitors,
248

selective direct factor Xa inhibitors,
244–248

prothrombinase complex, 86–87

thrombin signaling, 89–90

Cyclooxygenase-2 (COX-2), 138

Cytokine-induced neutrophil

chemoattractant-1 (CINC-1),
139, 140

Cytotrophoblastic cells (CTBs), 176

D

Dabigatran, 251

Deep vein thrombosis (DVT), 192

Diabetic foot ulcers, TP508, 126

Direct thrombin inhibitors (DTI), 250

Distal radius fracture repair, TP508, 127

DU-176b, FXa inhibitor, 247

E

Early growth response-1 (Egr-1) gene, 182

Endosomal-sorting complex required for

transport (ESCRT) machinery, 56

Endothelial cells

protection, thrombin

arterial protection, 104–105

endothelial cell survival, 104

PAR 1 activation, 103–104

vascular network formation, 105

Epidermal growth factor receptor (EGFR), 165

Epithelial malignancies

epithelia sheets morphogenesis, 174

- hPar1* transgenic mice
 - ablation of, 180
 - β -catenin stabilization, 178–179
 - mammary gland tissue, 177–178
 - survival factor, 180–181
 - transcriptional regulation, 181–184
 - Wnt-4 and wnt-7b, 178
 - placenta physiological invasion
 - EVT and CTBs, 176
 - and GTD, 177
 - protease-activated receptor1 (PAR₁)
 - overexpression
 - activation, 174
 - MCF10A breast cancer cells
 - morphogenesis, 175
 - Experimental autoimmune encephalomyelitis (EAE), 147, 148
 - Extravillous trophoblast (EVT), 176
- F**
- Focal adhesion kinase (FAK), 184
- G**
- Gastrointestinal tract proteinases, 25
 - Gestational trophoblastic disease (GTD), 177
 - G-protein-coupled receptor kinases (GRKs), 53
 - Growth-regulated oncogene (GRO), 139, 140
 - Growth-regulated oncogene-a (GRO-a), 163, 164
 - Growth-regulated oncogene/cytokine-induced neutrophil chemoattractant-1 (GRO/CINC-1), 139
 - Guanine nucleotide exchange factor (GEF), 50–51
- H**
- Hirudin inhibitor, 141
 - HIV and thrombin, 148–149
 - Human protease-activated receptor-1 (*hPar1*)
 - β -catenin stabilization, 178–179
 - mammary gland tissue, 177–178
 - as survival factor, 180–181
 - transcriptional regulation
 - androgen regulation, 181, 182
 - Egr-1 motif, 182–183
 - Matrigel invasion assay, 183
 - p53 tumor suppressor gene, 183, 184
 - transcription rate and mRNA stability, 182
 - wnt-4 and wnt-7b, 178
- 6-Hydroxydopamine (6-OHDA), Parkinson's disease, 147
- I**
- Idraparinux, 243–244
 - Immune-cell-derived proteinases, PAR regulation, 26–27
 - Inducible nitric oxide synthase (iNOS), 138
 - Interleukin, 138
 - Interstitial collagenase. *See* Matrix metalloproteinase
 - Ischemic brain, 140
- K**
- Kallikrein-related peptidases (KLKs)
 - prostate-specific antigen (PSA) and kallikrein 3, 27–28
 - tumour-related kallikreins, 28
- L**
- Lowmolecular-weight heparin (LMWH), 238
 - advantage, 197
 - vs. unfractionated heparin, 195
 - VTE treatment, 196
 - LY-517717, FXa inhibitor, 247–248
- M**
- Matrix metalloproteinase, 57
 - Metalloproteinase-9 (MMP-9), 142
 - Microglial cells, 138
 - Mitogen-activated protein kinase (MAPK), 138, 143
 - Multiple sclerosis (MS), 147–148
- N**
- Nematode anticoagulant proteins (NAPs), 240–241
 - Neuroinflammation
 - astrocytes, 139
 - GRO/CINC-1 release, 139, 140
 - microglial activation, 138
 - Nitric oxide (NO), 138, 139
 - N-methyl-d-aspartate (NMDA) receptors, 137, 143
 - Nonpeptide antagonists, PAR₁ modulators
 - E-5555 role, 216
 - FR171113, 214
 - himbacine, 217, 218

Nonpeptide antagonists, PAR, modulators (*cont.*)
 pyrroloquinazoline SCH-79797, 215
 Sch 530348, 219, 220
 urea-based and phenylisoxazole-based,
 214, 215
 Nuclear factor- κ B (NF- κ B), 138

O

Oral anticoagulants (OAC), 194
 Oxygen-glucose deprivation (OGD), 141

P

Parkinson's disease, 146–147
 Pathogen-derived proteinases, PAR activation,
 28–29
 Peptide agonists and antagonists, PAR₁
 modulators
 BMS-197525 C-terminus extension, 211
 3-mercaptopropionyl (Mpa) peptide,
 211
 structure-activity studies, 210
 TRAP-5 agonist peptide analogs, 209
 Placenta physiological invasion
 EVT and CTBs, 176
 and GTD, 177
 Protease-activated receptor1 (PAR₁), protein C
 pathway
 APC concentration, 73–74
 endothelial cell protein C receptor (EPCR),
 63–64
 membrane compartmentalization, 74–75
 PAR₁ cleavage, APC-EPCR, 73
 receptor activation kinetics, 74
 thrombomodulin, 64
 Protease-activated receptors (PARs)
 activation
 anti-coagulant protease activated
 protein C (APC), 56
 enzyme vs. peptide, 30–33
 inflammatory actions, 33–34
 plasmin and matrix metalloproteinase, 57
 protease factor Xa, 56
 proteolytic mechanism, 49
 and Alzheimer's disease, 144, 145
 apoptosis, 143
 cell-type-specific expression, 48
 expression, 134
 HIV-associated dementia, 148
 inflammatory responses, 33–34
 interactions
 conformational change, 11–12
 hirudin inhibitor, 9–10
 murine thrombin, 10–11

ischemia, 140
 and metalloproteinase-9 (MMP-9), 142
 multiple sclerosis (MS), 148
 neuroinflammation, 138
 PAR₁-induced RhoA activation, 135, 136
 and Parkinson's disease, 147
 PAR-mediated signalling, 29–30
 regulation

CNS proteinases, 25–26
 GI tract proteinases, 25
 immune-cell-derived proteinases,
 26–27
 kallikrein-related peptidase (KLK),
 27–28
 pathogen-derived proteinases, 28–29
 plasmin and activated protein C, 24
 Xa factor, 24–25

signaling

cell-type-specific responses, 51–52
 desensitization, 52–54
 down-regulation, 55–56
 heterotrimeric G-proteins, 50–51
 internalization, 54–55
 trypsin IV/mesotrypsin, 149, 151
 vasculogenesis, 89

Proteinase-activated receptor-4 (PAR₄) antagonists

cell-penetrating pepducin, 223
 peptide agonists, 222
 peptide and nonpeptide antagonists,
 222

Proteinase-activated receptor-1 (PAR₁) modulators

cell-penetrating pepducins, 220–221
 nonpeptide antagonists
 E-5555 role, 216
 FR171113, 214
 himbacine, 217, 218
 pyrroloquinazoline SCH-79797, 215
 Sch 530348, 219, 220
 urea-based and phenylisoxazole-based,
 214

peptide agonists and antagonists
 BMS-197525 C-terminus extension, 211
 3-mercaptopropionyl (Mpa) peptide,
 210, 211
 structure-activity studies, 210
 TRAP-5 agonist peptide analogs, 209
 peptidomimetic antagonists, robust
 solid-phase parallel syntheses
 RWJ-58259, 213, 214
 three-point model, 212
 tethered ligand, 208

Proteinase activated receptors

thrombin-tumor interactions, 162–164

- tumor cell
 - endothelial adhesion, 166–167
 - platelet interaction and adhesion, 165, 166
- Proteinases, PAR regulation
 - central nervous system, 25–26
 - gastrointestinal tract, 25
 - immune-cell-derived proteinases, 26–27
 - kallikrein-related peptidases (KLKs), 27–28
 - pathogen-derived proteinases, 28–29
- Protein C pathway, PAR₁
 - APC concentration, 73–74
 - endothelial cell protein C receptor (EPCR), 63–64
 - membrane compartmentalization, 74–75
 - PAR₁ cleavage, APC-EPCR, 73
 - receptor activation kinetics, 74
 - thrombomodulin, 64
- Protein kinase C (PKC), 138
- Prototypic anticoagulant/antithrombotic thrombin
 - acute thrombus formation, 13–14
 - enoxaparin and WE doses, 15
 - thrombin analog WE infusion, 14–15
- P-selectin, 166, 167
- p53 tumor suppressor gene, 183, 184
- Pulmonary embolism (PE), 192

R

- Razaxaban, 244
- Reactive oxygen species (ROS), 145
- Rivaroxaban
 - advantage, 246
 - in phase I studies, 245

S

- SNAC/heparin, indirect thrombin inhibitors, 249–250
- Sodium activation kinetics, thrombin
 - binding mechanism, 6
 - enthalpy change, 6–7
- SSR126517E, factor Xa inhibitors, 244
- Stroke
 - apoptosis, 143
 - and glutamate, 143
 - ischemic brain, 140
 - PAR₁ activation, 142, 143
 - TNF- α and iron, 142
 - TPC and brain edema, 141
- Suppressor of cytokine signaling 3 (SOCS3), 138

T

- Tau protein and Alzheimer's disease, 144
- TGN-167 inhibitor, 252
- Thrombin
 - allosteric conformations, 7–9
- APC
 - adhesion molecule expression, 67–68
 - apoptosis, 68–69
 - barrier integrity protection, 67
 - inflammation, 65
 - signaling, PAR, 65–66
 - surface retention, 75
- blood coagulation and vasculogenesis, 87–88
- characteristics, 19–20
- coagulation cascade
 - extrinsic pathway, 83–86
 - FX protein and nonenzymatic FV cofactor, 86
 - intrinsic pathway, 83
 - prothrombinase complex, 86–87
 - schematic representation, 82–83
 - thrombin signaling, 89–90
- endothelial cell protection
 - arterial protection, 104–105
 - endothelial cell survival, 104
 - PAR 1 activation, 103–104
 - vascular network formation, 105
- functions, 1–2
- non-PAR activation
 - fibrin agonist generation, 36
 - non-catalytic mechanisms, 35
 - non-receptor targets, 35
- PAR activation, inflammatory response, 33–34
- pro and anticoagulant activity dissociation, 12–13
- protease-activated receptors (PARs)
 - interaction
 - conformational change, 11–12
 - hirudin inhibitor, 9–10
 - murine thrombin, 10–11
- protein C pathway, PAR₁
 - APC concentration, 73–74
 - endothelial cell protein C receptor (EPCR), 63–64
 - membrane compartmentalization, 74–75
 - PAR₁ cleavage, APC-EPCR, 73
 - protein C interaction, 9
 - receptor activation kinetics, 74
 - thrombomodulin, 64
- proteolytic and nonproteolytic cell signaling

Thrombin (*cont.*)

- fibroblast proliferation, 116–117
- receptor cross-linking studies, 117
- seven transmembrane domain receptors, 117–118
- working model, 118
- prototypic anticoagulant/antithrombotic thrombin
 - acute thrombus formation, 13–14
 - enoxaparin and WE doses, 15
 - thrombin analog WE infusion, 14–15
- receptor identification
 - G-protein-coupled receptor superfamily, 20–21
 - non-PAR 1 receptor, 21–22
 - PAR-activating peptides, 21–24
 - thrombin-derived peptide and protease nexin, 20
- sodium activation kinetics
 - binding mechanism, 6
 - enthalpy change, 6–7
- sodium interaction and binding, 2–3
- structure
 - composition, 3–4
 - exosite I and II, 4–5
 - Thrombin X-ray structure, 5–6
- therapeutic implications
 - inflammatory process, 38
 - thrombin-targeted enzyme inhibitors, 37
 - thrombosis, 37–38
- thrombin-induced angiogenesis
 - coagulation-dependent pathways, 99–102
 - coagulation-independent mechanisms, 102–103
 - fibrin, 105
 - PAR 1 antagonists, 105–106
- thrombin receptors
 - PARs, 89
 - thrombomodulin (TM), 88–89
- TP508 peptide
 - angiogenesis, 121–122
 - binding specificity and photoaffinity cross-linking, 120–121
 - chemotactic activity, 121
 - diabetic foot ulcers, 126
 - distal radius fracture repair, 127
 - gene expression, 122–123
 - $\alpha v \beta 3$ integrin and thrombin modification, 120
 - location, 119
 - mitogenic activity, 118
 - proteolytic cleavage, 119–120
 - receptor-mediated endocytosis, 120

- tissue repair, 127–128
- wound healing, animal model
 - critical-sized defect model, 125
 - PAR 1-activating peptide (PAR 1-AP), 123
 - tissue repair, TP508, 124–125
- Thrombin preconditioning (TPC), 141
- Thrombin receptor modulators
 - PAR₁ and PAR₄, 206, 207
 - platelets activation and aggregation, 206
 - proteinase-activated receptor-4 (PAR₄)
 - antagonists
 - cell-penetrating pepducin, 223
 - peptide agonists, 222
 - peptide and nonpeptide antagonists, 222–223
 - proteinase-activated receptor-1 (PAR₁)
 - modulators
 - cell-penetrating pepducins, 220–221
 - nonpeptide antagonists, 214–219
 - peptide agonists and antagonists, 208–211
 - peptidomimetic antagonists, 212–213
 - therapeutic applications
 - anticancer therapeutics, 225–226
 - antithrombotic agents, 224
 - atherosclerosis and restenosis treatment, 224–225
- Thrombin receptors. *See also* Protease-activated receptors (PARs)
 - activation
 - anti-coagulant protease activated protein C (APC), 56
 - plasmin and matrix metalloproteinase, 57
 - protease factor Xa, 56
 - proteolytic mechanism, 49
 - cell-type-specific expression, 48
 - desensitization, 52–53
 - down-regulation, 55–56
 - internalization
 - adaptor protein complex-2 (AP-2), 54–55
 - arrestin-independent PAR 1 internalization, 54
 - ubiquitination, 55
 - signaling
 - cell-type-specific responses, 51–52
 - heterotrimeric G-proteins, 50–51
- Thrombomodulin (TM), 88–89
- Thromboprophylaxis
 - primary surgical thromboprophylaxis, 195–196
 - UFH and LMWHs, 194, 195
 - VTE prevention, 196

- Tissue factor (TF), 191
- Tissue factor pathway inhibitor (TFPI), 241–242
- Tissue plasminogen activator (tPA), 140
- TP508 peptide
- angiogenesis, 121–122
 - binding specificity and photoaffinity cross-linking, 120–121
 - chemotactic activity, 121
 - critical-sized defect model, 125
 - diabetic foot ulcers, 126
 - distal radius fracture repair, 127
 - gene expression, 122–123
 - $\alpha v \beta 3$ integrin and thrombin modification, 120
 - location, 119
 - mitogenic activity, 118
 - PAR 1-activating peptide (PAR 1-AP), 123
 - proteolytic cleavage, 119–120
 - receptor-mediated endocytosis, 120
 - tissue repair, 124–125, 127–128
- Tumor biology, thrombin role
- and cell dormancy
 - evidence for, 167, 168
 - thrombin role, 168 - and metastases
 - and metastasis mechanism, 167
 - PAR, activation, 165–167
 - supporting evidence, 164–165 - supporting evidence, 162
 - thrombin-tumor interactions mechanisms
 - angiogenesis induction, 163
 - chemokine growth-regulated oncogene- α (GRO- α), 163, 164
 - downstream mitogenic signaling, 162, 163
 - platelet-tumor interactions, 163 - thrombosis, 161, 162
- Tumor-derived proteinases. *See* Kallikrein-related peptidases (KLKs)
- U**
- Unfractionated heparin (UFH), 194, 238
- V**
- Vascular endothelial growth factor (VEGF), 163
- Vasculogenesis, thrombin
- coagulation cascade, 82–83
 - extrinsic pathway
 - Factor VII, 85–86
 - tissue factor, 83, 85 - FX protein and nonenzymatic FV cofactor, 86
 - gene mutations, 87–88
 - PARs, 89
 - prothrombinase complex, 86–87
- Venous thromboembolism (VTE), 161
- anticoagulant requirements, 253
 - cancer vs. non-cancer patients, 193
 - coagulation propagation inhibitors
 - factor XIa inhibitors, 248
 - indirect factor Xa inhibitors, 243–244
 - selective direct factor IXa inhibitors, 248
 - selective direct factor Xa inhibitors, 244–248 - phase II or III clinical studies, 239
 - prophylaxis for, 194
 - thrombin activity inhibitors
 - direct thrombin inhibitors, 250
 - selective oral direct thrombin inhibitors, 250–252
 - SNAC/heparin, 249–250 - tissue factor/factor VIIa complex inhibitors
 - anti-TF/VIIa, 241
 - nematode anticoagulant proteins, 240
 - TFPI, 241–242 - treatment of, 196
 - VKAs and UFH limitations, 238
- Vitamin K antagonists (VKAs), 238
- von Willebrand factor (vWF), 166
- VTE. *See* Venous thromboembolism
- W**
- Warfarin, 243, 244, 247
- Wound healing, thrombin
- critical-sized defect model, 125
 - proteolytic and nonproteolytic cell signaling
 - fibroblast proliferation, 116–117
 - receptor cross-linking studies, 117
 - seven transmembrane domain receptors, 117–118
 - working model, 118 - tissue repair, TP508, 124–125
 - TP508 peptide
 - angiogenesis, 121–122
 - binding specificity and photoaffinity cross-linking, 120–121
 - chemotactic activity, 121
 - diabetic foot ulcers, 126
 - distal radius fracture repair, 127
 - gene expression, 122–123

Wound healing, thrombin (*cont.*)
 α v β 3 integrin and thrombin
 modification, 120
 location, 119
 mitogenic activity, 118
 proteolytic cleavage, 119–120
 receptor-mediated endocytosis, 120
 tissue repair, 127–128

X

Ximelagatran, 250–253

Y

YM-150, FXa inhibitor, 247